Dissecting the Immune environment in Glioblastoma

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This thesis is dedicated to

Frances Ma, my parents

And

Prof. Vincenzo Cerundolo

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Declaration of Authorship

I declare that this thesis submitted entitled 'Dissection of the immune environment in glioblastoma' represents work performed by myself with the following exceptions:

The whole exome, mRNA sequencing analysis and neoantigen prediction was performed by Mr Isaac Woodhouse.

The bisulphite treatment and targeting sequencing was performed by Miss Sophie Kirschner and Mr Anandhakumar Chandran. The methylation sequencing analysis was performed by Miss Katherine Ferris.

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U87MG cell line was a gift from Prof. Valentine Macaulay.

The NHA cell line was a gift from Dr. Marta Pojo.

I declare that I have not submitted this work for any other degree.

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Abstract

Glioblastoma (GBM) is the most common primary brain tumour in adults. Unfortunately, it is also the most malignant, conferring a median survival of only 6 months, increasing to 14-24 months with maximal therapy. Despite much effort, there have been relatively little effective advances made for the treatment of GBM in the last decade. Immunotherapy, especially checkpoint inhibition, has revolutionised the management of some previously untreatable cancers, such as metastatic melanoma. However, such treatments have yet to be shown to have any significant effect for the treatment of GBM. This is in part due to the low tumour mutational burden and poor immunogenicity of this immune 'cold' tumour.

In this thesis I show that there are potential neoantigen encoding mutations, as well as a wide range of cancer testis antigens, that are not expressed or minimally expressed in GBM. Treatment of U87MG and GBM primary cell lines with the DNA methlytransferase inhibitor decitabine, leads to an increase in the expression of these immunogenic antigens due to hypomethylation. This increase in antigen expression leads to increased T cell recognition, activation and killing in a major histocompatibility complex – T cell receptor (TCR) dependent fashion. Further experiments are planned to translate these *in vitro* findings into an *in vivo* mouse model with a view to establishing a clinical trial.

Secondly, I also present single cell TCR sequencing data performed on primary and recurrent GBM samples from 13 patients. I show that the TCR diversity is much reduced in the tumour samples with many more expanded T cell clones. I further show that testing of the expanded TCR reveals some of these T cell clones are tumour reactive, but they are not the most significantly expanded clones. Further work is being undertaken to further characterise the tumour specific TCR identified in more detail as well as interrogate the clonal TCR seen in other patients.

1. Introduction

1.1. Immunology and Cancer

Humans are vastly outnumbered by microbial pathogens in nature and there remains a constant battle between us and these pathogens for survival. The immune system is our protection from these potentially harmful micro-organisms that are constantly evolving. In order to successfully recognise and eradicate any invading pathogen or toxin, both arms of the immune system, innate and adaptive, are able to distinguish self from non-self. As a by-product, our immune system is also able to distinguish between 'normal' healthy tissue and cancerous tissue. The role of the immune system in the surveillance and management of cancer is not a new concept. In 1891, William Coley, a surgeon, was the first to postulate that the immune system is capable of fighting cancer when he correlated the occurrence of postoperative infection with tumour eradication in a patient treated for sarcoma (Coley, 1891). He quickly followed up this finding with a series of patients' malignant tumours which he treated with repeated injections of streptococcus to induce erysipelas (Coley, 1893). Utilising these methods, he could achieve a cure rate of over 10%. Unfortunately, it seems William Coley was a century ahead of his time and, due to lack of knowledge and technology, the field of immunotherapy really stalled and quickly fell out of fashion. However, with increasing knowledge and some key breakthroughs, we are finally beginning to unleash some of this previously untapped potential.

1.1.1. Innate immune surveillance

The innate immune system is set up to recognise and react quickly to microbial infection and as such its activation and response is stereotyped (Janeway and Medzhitov, 2002). To achieve this, cells of the innate immune system predominantly sense microbial infection through toll-like receptors that recognise products of microbial metabolism that are not present in mammalian cells (Medzhitov, 2001). However, the innate immune system is also able to recognise cells that have been infected and compromised by microbial infection through recognition of signals of cellular stress (Muralidharan and Mandrekar, 2013) and cell death (Rock, Lai and Kono, 2011).

Cancer cells, by their nature are rapidly dividing cells with high energy requirements. This often causes endoplasmic reticulum (ER) stress (Verfaillie, Garg and Agostinis, 2013), cell necrosis with subsequent release of extracellular deoxyribonucleic acid (DNA) (Li *et al.*, 2003) as well as a whole host of other damage associated molecular patterns (DAMPs), which activates the innate immune system. Importantly, the innate immune system also acts synergistically to prime and enhance the adaptive immune system.

1.1.2. Adaptive immune surveillance

The adaptive immune system is a much more fine-tuned response that is capable of much more specific and nuanced recognition of non-self-antigens. The adaptive immune system consists of T, B, and NK lymphocytes. αβT cells, the main immune cell subset studied in this thesis, can recognize peptides, lipids, and vitamins presented on antigen presenting molecules, through their T cell receptor (TCR) and are thought to be the major players in cancer detection and eradication. In order to be able to recognise the immense number of

pathogenic antigens that we may encounter, the TCRαβ has evolved to be highly variable. It is a transmembrane complex containing 2 highly variable α and β chains that are linked by a disulphide bond (Garcia, Teyton and Wilson, 1999). Each α and β chain is a product of the somatic recombination of a variable (V) and joining (J) segment with an extra diversity (D) segment in the β chain (Clark *et al.*, 1984; Kee Sim *et al.*, 1984). There are multiple different copies of these regions in the genome, which can be variably rearranged to create huge variability (Yoshikai *et al.*, 1985). Each TCR chain has 3 hypervariable regions, of which the complementary determinant region 3 (CDR3) forms the centre of the binding pocket of the TCR. The CDR3 spans the joining of the VDJ regions, which allows the great variability in antigenic recognition.

Peptide antigens are presented on the major histocompatibility complex (MHC) I and II, which are polymorphic molecules with high variability in the population (Germain, 1994; Neefjes *et al.*, 2011). During an immune response, antigen presenting cells (APCs) present antigens and co-stimulate T cells through co-stimulatory molecules like CD28 and cytokine release which primes T cells (figure 1.1). Once primed, T cells can rapidly expand and migrate to tumours where they perform cytotoxic and cytokine-secreting (helper) functions. Self-reactive TCRs are filtered out during thymic selection and do not make it into the periphery (Takaba and Takayanagi, 2017). Therefore, the vast majority of epitopes presented on healthy cell surfaces should not induce an immune response. Upon activation, T cells upregulate expression of various markers such as PD-1, HLA-DR, CD39, CD103, TIM-3 and LAG-3 (Mohme *et al.*, 2018; Woroniecka *et al.*, 2018; Yost *et al.*, 2019; van der Leun, Thommen and Schumacher, 2020). Of these, CD103 has been reported to be a marker for tissue resident memory T cells , which may represent tumour reactive T cells and therefore play an important role in tumour eradication (Uss *et al.*, 2006; Duhen *et al.*,

2018; Hamid *et al.*, 2020; Hao *et al.*, 2020). CD39 is a T cell activation marker that has been reported to be specific for tumour reactive T cells in the context of cancer. $CD39^{+}$ CD4 and CD8 T cells are thought to represent an potential target for immunotherapy (Canale *et al.*, 2018; Duhen *et al.*, 2018; Kortekaas *et al.*, 2020).

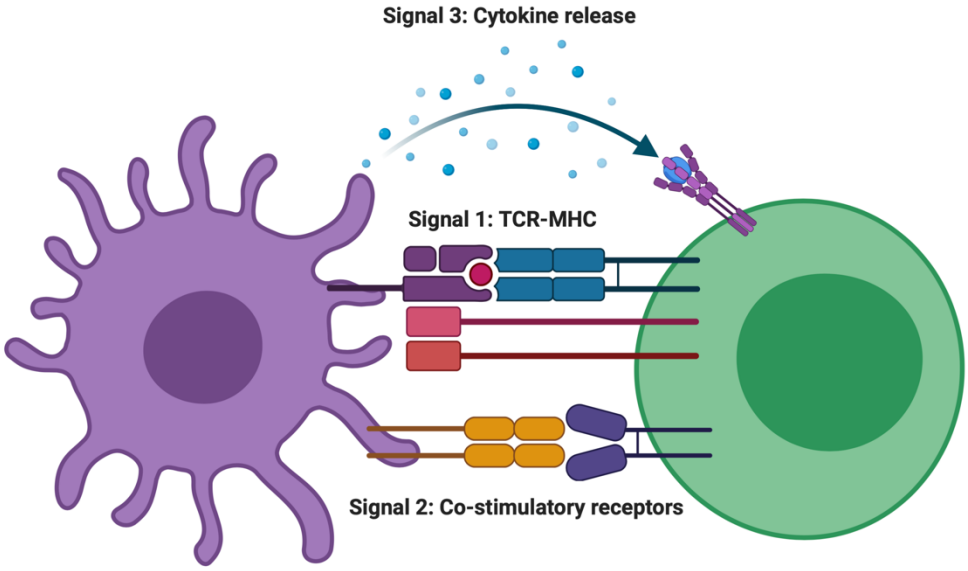


Figure 1.1 Mechanisms of T cell activation. Activation of T cells requires the presence of signal 1 – TCR:MHC engagement; signal 2 – co-receptor stimulation, such as CD28:CD80 or CD40:CD40L and signal 3 – cytokine stimulation, such as IL-2, IL-4, IL-7, IL-12 etc.

Other antigens like lipids and vitamins are presented on non-classical antigen presenting molecules such as the CD1 family and MR1 (Brigl and Brenner, 2004; Huang *et al.*, 2005; Mori, Lepore and De Libero, 2016). Within these, there are a subset of unconventional, mucosal-associated invariant T cells (MAIT cells). MAIT cells are activated by bacterial metabolites from the vitamin B2 synthesis pathway presented on a non-polymorphic MHC class I-related (MR1) molecule (Kjer-Nielsen *et al.*, 2012; Corbett *et al.*, 2014). Classically, they were identified by their invariant Vα7.2–Jα33 chain paired with a limited β chain, although more recently there are other non-classical MAIT cells with other α chains

described (Godfrey *et al.*, 2019). Upon activation, MAIT cells are able to produce pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and Interferon- γ (INF- γ) as well as have direct cytotoxic activity (Godfrey *et al.*, 2019). As such, they have been described as a bridge between the innate and adaptive immune system. Given their specificity to bacterial metabolites, MAIT cells have been found to play a key role in response to bacterial infections (Godfrey *et al.*, 2019; Toubal *et al.*, 2019). More recently, MAIT cells have also been found to be present in certain cancers such as colorectal cancers (Zabijak *et al.*, 2015; Ling *et al.*, 2016; Li *et al.*, 2020), hepatocellular carcinomas (Duan *et al.*, 2019), hepatic metastases (Shaler *et al.*, 2017) as well as renal and brain tumours (Peterfalvi *et al.*, 2008). Their potential relevance in mucosal-associated tumours is understandable but less so for other cancers, such as glioblastoma tumours. In addition, it is unclear whether their presence is beneficial with some evidence to suggest that they correlate with a negative prognosis (Zabijak *et al.*, 2015; Duan *et al.*, 2019) and others suggesting that they may have an anti-cancer function (Flores-Villanueva *et al.*, 2020; Li *et al.*, 2020).

1.1.3. Cancer antigens

The adaptive immune system, due to its ability to detect foreign antigens with high fidelity can play a key anti-cancer role. This is because, cancers can present antigens that the adaptive immune system has not encountered before, or only in small quantities and therefore T cells reactive to these antigens have not undergone thymic selection. These antigens can be broadly classified into three categories: 1) cancer testis antigens (CTA), which are genes which are usually only expressed in the testis but can be re-expressed in

cancers (Chinnasamy *et al.*, 2011; Freitas *et al.*, 2013; Shukla *et al.*, 2018; Lajer *et al.*, 2019). 2) tumour associate antigens (TAAs) are either mutations that are commonly present in specific cancers, such as the endothelial growth factor receptor variant III mutation (EGFRvIII) detected in roughly 30% of glioblastoma (GBM) (Wikstrand *et al.*, 1997) or genes that are overexpressed in cancers as compared to normal tissue such as human epidermal growth factor receptor 2 (HER2), which is overexpressed in 30-40% of breast cancers (Brossant *et al.*, 1998). 3) neoantigens are patient specific antigens that usually arise from DNA mutations within cancers. The process of oncogenesis involves several mutagenic steps, usually in oncogenes or tumour suppressor genes, many of which are involved in DNA repair. Because of this, cancers accumulate many mutations (Alexandrov *et al.*, 2013, 2020) some of which can lead to changes in the peptide sequences of proteins. If the mutated peptide sequences are presented on major histocompatibility complex (MHC), they can potentially be recognised as non-self and elicit a T cell response (Schumacher and Schreiber, 2015; Schumacher and Hacohen, 2016).

1.1.4. Cancer immune escape

Despite all of the potential avenues for the immune system to target and eradicate them, cancers still occur and are becoming more common. The incidence of cancers has increased 5% in the last decade to over 367,000 new cases a year between 2015-2017 (Cancer Research UK statistics, https://www.cancerresearchuk.org/health-professional/cancer-statistics/incidence#heading-Zero). This is because cancer constantly evolves to evade immune surveillance. To overcome T cell mediated cytotoxicity, it is well documented that many tumours undergo downregulation of their antigen presentation machinery leading

to reduced peptide presenting MHC molecules on the surface and this reducing recognition by TCRs (Burr *et al.*, 2019). Furthermore, cancer cells can also acquire mutations and loose expression of HLA alleles (McGranahan *et al.*, 2017; Rosenthal *et al.*, 2019) thereby not only decreasing the amount of peptide presented but also the range of peptides that can be presented. Loss of beta-2 microglobulin (B2M), a more drastic event that prevents the presentation in MHC-I in general, has also been described in several tumours especially in tumours with a high mutation rate and therefore potentially high numbers of neoantigens (Gettinger *et al.*, 2017; Grasso *et al.*, 2018; Castro *et al.*, 2019). Even if the antigens are presented and recognised by T cells, tumours have evolved methods to reduce the activity of these T cells. Examples of this include secretion of many immunosuppressive cytokines such as transforming growth factor β (TGF-β), prostaglandin-E2 (PDG-E2) and interleukin (IL)-10/6 leading to immunosuppressive macrophage (M2-like) and regulatory T cell polarisation (Rabinovich, Gabrilovich and Sotomayor, 2007) as well as inducing immune tolerance through changes in amino acid metabolism (Timosenko, Hadjinicolaou and Cerundolo, 2017). In addition, tumours are able to reduce the activation and effector potential of cytotoxic T cells within the tumour environment through expression of immunomodulatory molecules such as programmed cell death ligand 1/2 (PD-L1/2).

In the rest of this chapter I will explore the various immunotherapeutic strategies currently utilised to overcome this immune resistance of tumours for the treatment of cancers. I will also focus on glioblastoma (GBM) and highlight the issues preventing the more widespread adoption of such treatments.

1.2. Recent advances in immunotherapy for cancers

1.2.1. Cytokine therapy

It was discovered in the 1980s that the stimulation of peripheral blood mononuclear cells (PBMC) from cancer patients could lead to the polarisation of a subset of T cells that were not specific to tumours in a classical TCR-MHC dependent fashion but were capable of inducing tumour lysis. These cells were termed lymphokine activated killer cells (LAK). This sparked a wave of enthusiasm towards the use of these cells either by adoptive cell transfer of autologous LAK cells or through injection of interleukin-2 (IL-2) to stimulate these cells *in vivo*. Unfortunately, despite some success is small trials (Dutcher *et al.*, 1989; Weiss *et al.*, 1992), a lack of success in large randomised trials, coupled with the high rate of severe toxicity associated with high dose IL-2 injection meant this technique has fallen out of favour (Négrier *et al.*, 1992; Law *et al.*, 1995; Hidalgo *et al.*, 1996).

1.2.2. Adoptive cell transfer

Classically, adoptive cell transfer trials have tended to use autologous T cells isolated and expanded from the tumour infiltrating lymphocyte (TIL) fraction. The rationale behind this was that there would be a subset of TIL that were tumour reactive and the lack of tumour control *in vivo* was down to the low numbers of these T cells and the immunosuppressive environment where they were embedded. Therefore, if it was possible to increase the number of tumour specific T cells and reinvigorate them then hopefully there would be a much greater effect on tumour control. Initial studies were performed using unselected expanded TIL infusions predominantly from patients with metastatic melanoma with some success (Dudley *et al.*, 2005; Hong *et al.*, 2010; Andersen *et al.*, 2016). However, these

techniques used lymphodepleting protocols prior to therapy and high dose IL-2 in order to maintain these T cells post treatment with all the inherent toxicities. A more recent study suggests that this may be feasible with lower doses of IL-2 and therefore be better tolerated (Nguyen *et al.*, 2019). Given the unselective nature of TIL expansion, initially researchers were unsure whether the T cells infused back into the patient were in fact tumour reactive. Reports to investigate this have shown that there are tumour reactive T cells in the infused products from patients that do respond to this treatment and some of the T cells may in fact be neoantigen specific and has been shown to sufficient to induce a long term anti-tumour effect in the treatment of both metastatic melanoma and breast cancer (Prickett *et al.*, 2016; Zacharakis *et al.*, 2018). However, the proportion of tumour reactive T cells in the infused products are still low but there are methods being developed to select and transfuse only the relevant T cells back into the patients (Kato *et al.*, 2018; Ahmadzadeh *et al.*, 2019). In addition, it is possible to identify tumour reactive T cells in the blood of such patients, potentially offering a route to less invasive techniques for generation of tumour reactive T cell infusions (Gros *et al.*, 2016; Cafri *et al.*, 2019).

Conventional T cells by their very nature can only recognise peptides presented on MHC molecules and as mentioned earlier, some tumours either downregulate or undergo HLA loss of heterozygosity as immune escape mechanisms. Adoptively transferring classical T cells in this situation would not mediate an effect if the epitope is not presented. Chimeric antigen receptors T cells (CAR-T) can overcome this issue. CAR-T cells are engineered to express single chain receptors that have an antibody-like antigen binding domain linked to an intracellular T cell activating domain. They are therefore like antibodies in the way they recognize antigens, in an MHC-independent manner, but they are also able to directly lead to T cell activation rather than relying on antibody dependent cell-mediated cytotoxicity

(ADCC). CAR-T cells have been shown to be especially effective in liquid tumours with CD19 CAR-T cell therapy using Tisagenlecleucel licensed for the treatment of B cell lymphoma since 2017 (Maude *et al.*, 2018; Schuster *et al.*, 2019). However, their application in solid tumours is much less effective due to the higher numbers of obstacles to overcome in solid tumours such as CAR-T cell trafficking to and into solid tumours as well as overcoming the immunosuppressive microenvironment that is less of an issue in blood cancers (Newick *et al.*, 2017). Furthermore, by their very nature, CAR-T cells are only able to bind to and respond to cell surface antigens. Unfortunately, there are very few known tumour specific cell surface antigens to target in solid tumours and those that are potential targets, such as EGFRvIII or IL13Rα2 are not present on all cancer cells (Wikstrand *et al.*, 1997), leading to the potential for tumour escape.

1.2.3. Oncolytic viruses

Oncolytic virus therapies utilise native or genetically modified viruses that selectively infect and replicate within tumour cells. The ability of viruses to kill tumours has been recognised since the late 1940's (Moore, 1949, 1951) but, similar to the field of onco-immunology as a whole, clinical translation has only borne fruit in the last few decades as we obtained a better understanding of viral, immune and tumour biology and their interplay. There are many classes of viruses that have been trailed and tested including, but not limited to, adenovirus, herpesvirus (HSV), poliovirus, vaccinia virus, coxsackie virus, parvovirus, reovirus, measles virus and Newcastle disease virus (Kaufman, Kohlhapp and Zloza, 2015). The ability of these viruses to preferentially target and replicate in cancer cells is thought to be a consequence of the cancer cells adaptation to escape the host immune system. In

normal cells, viral infection initiates local interferon (IFN) release from infected cells and activation of the Janus kinase–signal transducer and activator of transcription (JAK-STAT) pathway to promote viral clearance. This has found to be aberrant in many cancer cells (Clemens, 2004). Viruses can also be relatively easily manipulated to delete non-essential virulence genes to enhance replication by removing the need to copy unnecessary genomic material and/or insertion of tissue specific promoters to guide gene expression in tumour cells (Vile and Hart, 1994).

The mechanisms behind how oncolytic viruses work are incompletely understood and vary between viruses based on their ability to replicate, tropism for cancer cells and the susceptibility of cancer cells to the different forms of cell death. In general, it is thought that oncolytic viruses are able to induce immunogenic cell death through induction of endoplasmic reticulum (ER) stress and release of damage-associated molecular patterns (DAMPs), as well as directly producing pathogen-associated molecular patterns (PAMPs), which can activate the innate immune system (Medzhitov, 2001; Muralidharan and Mandrekar, 2013). Cell death will also release soluble antigens, which can be taken up by professional APCs, such as dendritic cells (DC), which have been attracted by activation of the innate immune system. This can lead to cross-priming and activation of the adaptive immune system. Cell death may indeed release new antigens that were previously unprocessed and presented by cancer cells or direct presentation may have led to poor T cell activation. Cross presentation is much more effective at generating an effective immune response and leads to a broader response through antigen spreading (Nesslinger *et al.*, 2010; Woller *et al.*, 2015; Gulley *et al.*, 2017). Although the initial immune response will be viral targeted, this can then lead to further response against the tumour directly. Viruses can also be altered to deliver transcripts encoding for cytokines that can enhance

immune responses (Passer *et al.*, 2013; Tähtinen *et al.*, 2016) or co-stimulatory molecules necessary for T cell activation (DiPaola *et al.*, 2006; Zamarin *et al.*, 2017).

The key for successful oncolytic viral therapy requires the fine balance between persistent viral replication within cancer cells and an active host immune response to clear infected cells. If virus replication is greater than the immune response then it may lead to an uncontrolled viral infection and damage to healthy tissues and conversely, an overzealous immune response will clear the virus infection quickly leading to ineffective anti-tumour response (Zamarin *et al.*, 2017). This balance is often quite hard to achieve, especially since the treatment is a 'live' treatment and may behave differently in different patients leading to variable effective doses. Often multiple treatments are necessary, and the host immune system may develop virus-specific neutralising antibodies against them over subsequent doses leading to increasingly ineffectual responses (Lauer *et al.*, 2018). However, there have been some successful trials, notably a phase III trial of Talimogene laherparepvec (T-VEC), which is a modified HSV1 based oncolytic virus against advanced melanoma. There was a durable response rate (16.3% vs. 2.1%) compared to Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) with a modest increase in median survival (23.3 months vs. 18.9 months) (Andtbacka *et al.*, 2015). Following this clinical trial, T-VEC is currently the only oncolytic virus that has achieved Food and Drug Agency (FDA) approval.

1.2.4. Vaccination

Vaccinations have been used successfully against pathogens since the 1790s when Edward Jenner created the first ever vaccination against smallpox and were the first class of immunotherapy trialled for cancer by William Coley in the 1890s. However, there still

remains few clinically approved cancer vaccines available today. With the help of ever more sophisticated sequencing techniques to identify more tumour antigens, including CTA, TAA and personalised neoantigens, as well as better knowledge and tools to overcome the immunosuppressive tumour microenvironment, there is increasing evidence that cancer vaccines may play an important role in the fight against cancer (Cecco *et al.*, 2010). Vaccines can broadly fall into three major categories: 1) peptide; 2) genetic (Ribonucleic acid (RNA) or DNA) or 3) cellular and I will briefly discuss the relative merits of each.

The simplest form of cancer vaccine that exists today is the peptide/protein vaccine. They are relatively cheap to produce, store and deliver on a mass scale and can consist of a single, or multiple peptides. Many peptide vaccines have undergone trials with demonstration of enhanced immune responses but changes in survival following treatment still remain elusive. This is likely due to the fact many of these vaccines contain only a single antigen, allowing for tumour escape through downregulation of the antigen (Dunn, Old and Schreiber, 2004; Mittal *et al.*, 2014). In addition, most peptides used are short peptides (15 amino acids or less), which do not need processing and are presented readily on MHC class I present on the majority of cells, not just APCs. However, non-APCs lack the relevant co-stimulatory factors required for optimal T cell priming and, in the absence of these, priming is suboptimal and can lead to dysfunctional T cells and an ineffectual immune response (R. E. M. Toes *et al.*, 1996; R. E. Toes *et al.*, 1996; Hailemichael *et al.*, 2013). In addition, short peptides are not presented on MHC class II molecules and cannot elicit a CD4 T helper cell response, which is known to be essential for optimal CD8 T cell activity. To overcome some of these issues, longer peptides have been used, which can prime both CD4 and CD8 T cell responses, although these peptides require processing

by APCs (Janssen *et al.*, 2005; Bijker *et al.*, 2008; Zhang *et al.*, 2009). All peptides, short or long, do not elicit a significant inflammatory response by themselves and often require an adjuvant to be given alongside to boost the response. Adjuvants such as the toll-like receptor (TLR) 3 ligands, polyinosinic:polycytidylic acid (Poly-IC) and polyinosinic-polycytidylic acid-poly-L-lysine carboxymethylcellulose (Poly-ICLC) have been proven to be effective and are often included in peptide vaccines in clinical trials (Zhu *et al.*, 2007; Migliorini *et al.*, 2019).

Similar to peptide vaccines, DNA/RNA vaccines are relatively simple and cheap to manufacture. They have the added benefit of being able to also include trip nucleic acid sensors that activate DCs, including certain TLRs, stimulator of interferon genes (STING), absent in melanoma 2 (AIM2), and DNA-dependent Activator of Interferon-regulatory Factors (DAI) pathways reducing the need for adjuvants. Free DNA/RNA can be taken up by APCs but the efficiency is low and other adjunctive measures, including electroporation, nanoparticle delivery or microneedle arrays have been trialled with better success (Jorritsma *et al.*, 2016). Such vaccines have been used with some success in the clinic with a phase IIb trial in cervical intraepithelial neoplasia showing increased rates of tumour regression (48.2% vs 30%) (Trimble *et al.*, 2015). A follow up phase III trial is currently underway (NCT03185013). RNA vaccines offer advantages over DNA vaccines in that they do not integrate into the genome and therefore have no oncogenic potential and they only need to enter the cytoplasm for effect compared to the nucleus. However, they are more unstable than DNA and susceptible to depredation by ubiquitous RNases. Recently, a completely personalised RNA vaccine approach for melanoma has been trialled with successful induction of neoantigen T cell responses all 13 patients (Sahin *et al.*, 2017). Alternatively, both RNA/DNA can be delivered in a viral vector similar to oncolytic viruses.

This approach was used for the phase II PROSTVAC trial of vaccinia virus/fowlpox virus encoding prostate specific antigen (PSA) for the treatment of metastatic castration-resistant prostate cancer where there was a 10 month median survival advantage (Kantoff, Gulley and Pico-Navarro, 2017). However, this was not repeated in a larger phase III trial.

Finally, cell-based vaccines are the most complex to produce and deliver but offer the potential for greatest coverage of potential immunogenic peptides without the need for screening. They can consist of either irradiated/killed cancer cells or DCs loaded with antigens from cancer cells. The GVAX vaccines are whole tumour cells modified to also secrete GM-CSF to promote local inflammatory response. Although there was success in pre-clinical models, translation into the clinic was less successful despite the presence of an increased immune response (Salgia *et al.*, 2003; Small *et al.*, 2007; Laheru *et al.*, 2008; Lipson *et al.*, 2015). There were high hopes for autologous DC vaccines following the FDA approval of sipuleucel-T in 2010 (DCs stimulated with GM-CSF and antigen PAP) for the use in metastatic castration resistant prostate cancer (Kantoff *et al.*, 2010). However, there have been no further approvals to date although there are a few small trials that would suggest tumour lysate pulsed DCs may offer some clinical benefit in metastatic melanoma and renal cell carcinoma (Zheng *et al.*, 2015; Bol *et al.*, 2016). This approach has also been used in glioblastoma, as I will discuss later in the chapter.

1.2.5. Immune checkpoint inhibition

First discovered in 1992 and 1994, programmed cell death 1 (PD1) and cytotoxic T lymphocyte–associated protein 4 (CTLA4) are 2 key immune regulatory checkpoints that modulate T cell activity (Ishida *et al.*, 1992; Walunas *et al.*, 1994; Leach, Krummel and

Allison, 1996; Okazaki *et al.*, 2013). The discovery that inhibition of these pathways can lead to reversal of T cell exhaustion and anergy in tumours has revolutionised the management of some previously untreatable cancers and has earned Tasuku Honjo and James Allison the Nobel prize for medicine in 2018 for their role in the discovery and utilisation of checkpoint inhibitors.

CTLA-4 is a negative regulator of T cell activation. It translocates to the T cell membrane where it competes with CD28 for the B7 ligand inhibiting proliferation and IL-2 secretion by T cells (Krummel and Allison, 1995). In contrast, PD1 is a negative regulator of T cell effector function in the periphery (Okazaki *et al.*, 2013). It is upregulated in T cells, B cells and monocytes upon activation (Agata *et al.*, 1996). Upon binding to ligand PD-L1 or PD-L2, PD1 limits lymphocyte proliferation and cytokine secretion (Freeman *et al.*, 2000) to prevent excessive immune-mediated damage following activation of an immune response (figure 1.2).

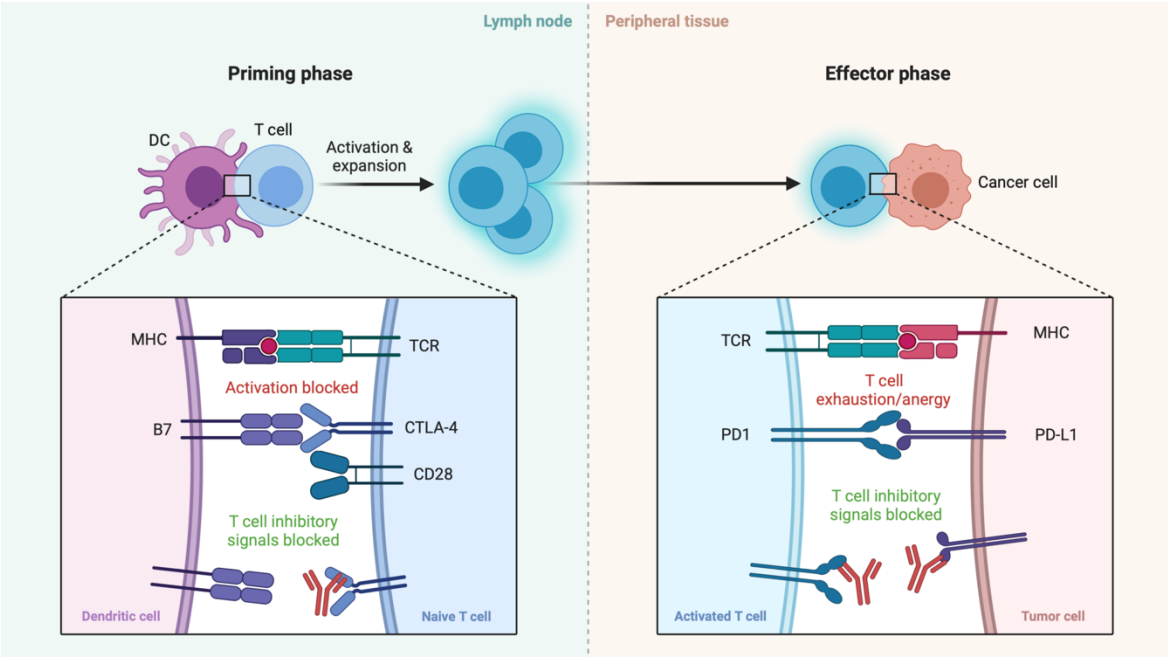


Figure 1.2 Mechanism of action of immune checkpoints CLTA-4 and PD1 and action of therapeutic blocking antibodies.

Cancers have been shown to hijack these physiologically relevant systems as a method of immune escape. Therapeutic antibodies against CTLA4 (Ipilimumab (Hodi *et al.*, 2008) and Tremelimumab (Ribas *et al.*, 2005)); PD1 (Nivolumab (Topalian *et al.*, 2012) and Pembrolizumab (Schachter *et al.*, 2017)); and PD-L1 (Durvalumab (Powles *et al.*, 2017) and Atezolizumab (Rosenberg *et al.*, 2016)) have produced remarkable results in the management of certain cancers, leading to durable responses in over 50% of patients with metastatic melanoma (Wolchok *et al.*, 2013). Their success has meant that in a relatively short time they are now key treatment options for many cancers including, but not limited to, metastatic melanoma (Wolchok *et al.*, 2013; Topalian *et al.*, 2014; Larkin *et al.*, 2015; Robert *et al.*, 2015; Schadendorf *et al.*, 2015), non-small cell lung cancer (Borghaei *et al.*, 2015; Brahmer *et al.*, 2015; Garon *et al.*, 2015; Rizvi *et al.*, 2015; Herbst *et al.*, 2016; Reck *et al.*, 2016) and renal carcinoma (Motzer, Escudier, *et al.*, 2015; Motzer, Rini, *et al.*, 2015).

More recently, other immune checkpoints such as T cell immunoglobulin and mucin domain-containing protein 3 (TIM3) (Wolf, Anderson and Kuchroo, 2019), T cell immunoreceptor with Ig and ITIM domains (TIGIT) (Manieri, Chiang and Grogan, 2017) and lymphocyte-activation protein 3 (LAG3) (Goldberg and Drake, 2011) have been identified as targets for potential therapy. Anti-TIM3 antibodies have shown the most promise, at least in mouse models, in combination with existing checkpoint inhibitors (Ngiow *et al.*, 2011). Currently there are many clinical trials underway to investigate their use for the treatment of various cancers including use of anti-TIM3 (NCT02817633, NCT03489343), anti-LAG3/PD1 (NCT04140500, NCT01968109), anti-TIGIT/LAG3 (NCT04150965) and anti-TIGIT/PD1 (NCT04047862). Conversely, co-stimulatory check points have also been discovered such as CD137 (4-1BB), glucocorticoid-induced tumour necrosis factor receptor (GITR) and CD40. Stimulatory antibodies against these checkpoints have also been

developed therapeutically and many are currently undergoing early phase trials. Of note, the anti-4-1BB antibody Utomilumab (Tolcher *et al.*, 2017) in combination with Pembrolizumab and anti-GITR antibody (Koon *et al.*, 2016) have been shown to be safe and tolerated in a phase I trials. Further studies looking at their efficacy are currently eagerly awaited.

1.3. Glioblastoma

Glioblastoma (GBM) is the most common and, unfortunately, the most malignant brain tumour in adults. Each year in the UK more than 4,000 new cases of central nervous system (CNS) cancers are diagnosed, which equates to approximately 7 per 100,000 population. Although brain tumours account for less than 2% of all primary tumours they are responsible for 7% of the years of life lost from cancer before age 70 (Office of National Statistics 2006 Series MB1 No. 34). More people under the age of 40 die from a brain tumour in the UK than any other form of cancer and 65% more women die from a brain tumour than from cervical cancer (Office of National Statistics, Mortality Statistics: Deaths registered in 2007). This high mortality rate means that this rare tumour is actually the third leading cause of cancer-related death among economically active men between 15-54 years of age and the fourth leading cause of cancer-related death among economically active women between 15-34 years of age (Kesari and Stiles, 2006). If the burden of disease is considered in terms of the average years of life lost per patient, brain tumours are one of the most lethal cancers with over 20 years of life lost (Burnet *et al.*, 2005). Unfortunately, despite significant research into these tumours, the latest survival trends for patients with

CNS malignancies have remained largely static (Rachet *et al.*, 2008) reflecting the lack of therapeutic options for patients.

The current gold standard of treatment involves gross total resection (GTR, as defined by complete resection of contrast enhancing tumour on a postoperative scan performed within 72 hours of surgery) of the tumour followed by concurrent radio-/chemotherapy with temozolomide and subsequent adjuvant temozolomide chemotherapy (Stupp *et al.*, 2005). Despite optimal treatment the median survival for such patients is still only 14-24 months and a five-year survival of approximately 10% (Stupp *et al.*, 2005; Stummer *et al.*, 2012). More recently, many clinical studies have been performed, all with limited success, looking at utilisation of other adjuvant therapies such as, the vascular endothelial growth factor (VEGF) inhibitor Bevacizumab (Chinot *et al.*, 2014; Gilbert *et al.*, 2014; Poulsen *et al.*, 2014; Heiland *et al.*, 2016; Schaub *et al.*, 2016), the integrin inhibitor Cilengitide (Stupp *et al.*, 2014) intra-operative chemotherapy through the use of Gliadel wafers (Westphal *et al.*, 2006; Attenello *et al.*, 2008; Barr and Grundy, 2012; Chowdhary, Ryken and Newton, 2015; Xing *et al.*, 2015) as well as both cell and non-cell based immunotherapy (Yamanaka *et al.*, 2003; Kunwar *et al.*, 2010; Stragliotto *et al.*, 2013; Ampie *et al.*, 2015; Antonios, Everson and Liau, 2015; Preusser *et al.*, 2015; Chow *et al.*, 2015; Johanns *et al.*, 2016, 2017; Brown *et al.*, 2016; Tabori *et al.*, 2016; Finocchiaro and Pellegatta, 2016; O'Rourke *et al.*, 2017; Omuro *et al.*, 2017; Saha, Martuza and Rabkin, 2017; Keskin *et al.*, 2018; Lang *et al.*, 2018; Samson *et al.*, 2018; Desjardins *et al.*, 2018; Hilf *et al.*, 2018; Iorgulescu *et al.*, 2018; Sampson *et al.*, 2019; Schalper *et al.*, 2019; Weller and Le Rhun, 2019; Chiocca *et al.*, 2019; Cloughesy *et al.*, 2019). Here, I will dissect our current knowledge of the tumour microenvironment and discuss the immunotherapeutic modalities trialled in GBM in depth.

1.3.1. The tumour immune microenvironment

The brain has long been thought to be an immune 'privileged' organ, kept separate from the rest of the body through the blood brain barrier. This was supported by the lack of a lymphatic system and a relative absence of antigen presenting cells (Forrester *et al.*, 2008; Louveau, Harris and Kipnis, 2015). However, the discovery of a 'glymphatic' system, that is capable of draining brain extracellular fluid and other macromolecules to the superior cervical lymph nodes, has cast doubt on this previously held dogma (Aspelund *et al.*, 2015; Louveau *et al.*, 2015). We now know that T cells are able to infiltrate the brain (Sampson *et al.*, 2019), especially in GBM where blood brain barrier is disrupted (Long, 1970; Watkins *et al.*, 2014). The brain is however unique in that it harbours a large resident population of myeloid cells composed, in the absence of inflammation, almost entirely of microglia (Li and Barres, 2018). In addition, brain tumours have also been shown to proffer their own methods of immune escape such as sequestration of T cells within the bone marrow, preventing effective immune surveillance and fostering antigenic ignorance (Li and Barres, 2018). Therefore, many people now propose that the brain is immunologically 'distinct' offering up its own unique set of obstacles to immune-based treatment of cancers arising within.

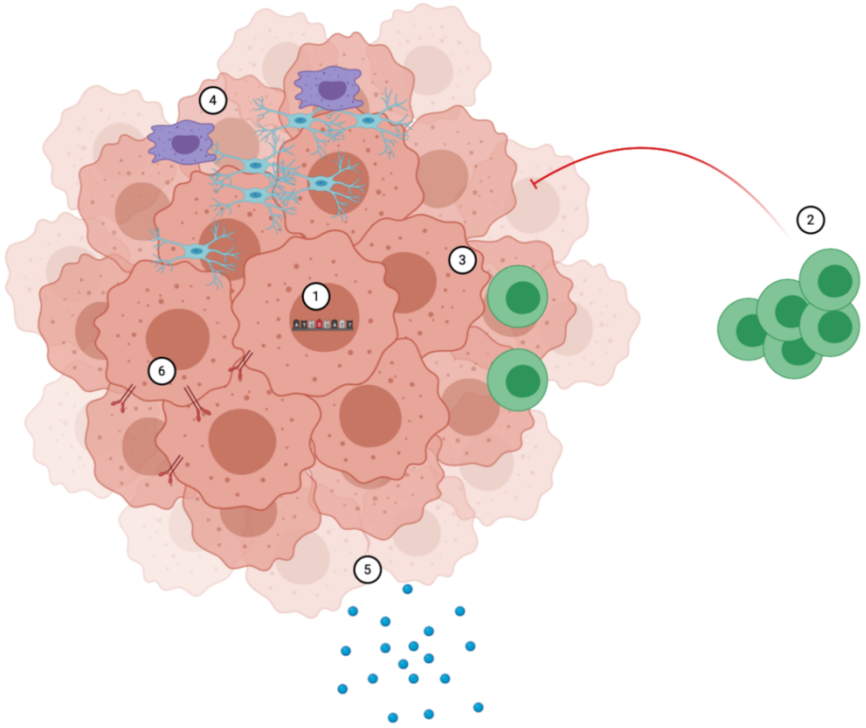


Figure 1.3 Mechanisms of immune evasion in glioblastoma. 1 – GBM have a low tumour mutational burden; 2 – T cells do not infiltrate into the tumour; 3 – T cells that are present are severely exhausted or Tregs; 4 – there are a high number of immunosuppressive microglia and M2 macrohpages; 5 – GBM secrete many immunosuppressive cytokines; 6 – GBM can also express PDL-1.

GBM is a classically immune 'cold' tumour (figure 1.3), harbouring a low tumour mutational burden (Schumacher and Schreiber, 2015) with only 0.8-3.5% of primary GBM harbouring a hypermutated genotype (Erson-Omay *et al.*, 2015; Wang *et al.*, 2016; Hodges *et al.*, 2017). Immune editing has been described for cancers where the immune response shapes the antigenic landscape of the tumour over time and treatment (Mittal *et al.*, 2014). This has also been shown to be the case in GBM (Barthel *et al.*, 2019) highlighting the potential that immunogenic antigens are gradually silenced over time.

GBM contains very few infiltrating immune effector cells (Gajewski, Schreiber and Fu, 2013). Analysis of histopathological slides from 171 GBM patients in The Cancer Genome

Atlas (TCGA) revealed that more than half (54%) of tumours had no detectable lymphocytes, 35% had immune cells in less than half of the tumour, and only 11% had lymphocytes detected in more than half of the tumour (Caleb Rutledge *et al.*, 2013). Of these TIL the vast majority have been found to be severely exhausted and incapable of mounting an effective anti-tumour response (Woroniecka *et al.*, 2018). There are also a substantial subset of CD4 regulatory T cells (Tregs) that are attracted to the GBM microenvironment through secreted cytokines such as CCL22 (Crane *et al.*, 2012). These Tregs, whose relative abundance can go as high as 60% of all TILs, act to dampen the immune response in GBM and are a significant prognostic indicator (Heimberger *et al.*, 2008; Yue *et al.*, 2014; Sayour *et al.*, 2015). In addition to a low number of exhausted TILs and presence of Tregs, GBM also harbour a vast number of immunosuppressive myeloid cells that can compose 30-50% of the tumour (Rossi *et al.*, 1987). This population is predominantly made up of microglia, but macrophages and myeloid-derived suppressor cells (MDSC) are also recruited to the tumour site by chemokines secreted by GBM including C-C motif chemokine ligand 2 (CCL2), C-C motif chemokine ligand 7 (CCL7), colony stimulating factor 1 (CSF-1), hepatocyte growth factor (HGF), C-X3-C motif chemokine ligand 1 (CX3CL1), adenosine triphosphate (ATP), dehydrogenase/reductase 3 (DHR3), glial cell-derived neurotrophic factor (GDNF) and GM-CSF (Chang *et al.*, 2016; Hambardzumyan, Gutmann and Kettenmann, 2016). The relative role of these different myeloid cells is not fully elucidated but it is clear that there is a synergistic interplay between them and GBM cells to promote their recruitment, polarisation into immunosuppressive subtypes and subsequent tumour progression (Wu *et al.*, 2010; Sarkar *et al.*, 2014; Zhou *et al.*, 2015). Therapeutically blocking immunosuppressive M2 polarisation can reverse this effect (Pyonteck *et al.*, 2013).

GBM also secrete a cocktail of immunosuppressive cytokines that inhibit both T cell migration and also subsequent activation including transforming growth factor β (TGF-β), prostaglandin E2 (PDG-E2), interleukin 10 (IL-10), vascular endothelial growth factor (VEGF) as well as enzymes such as Indoleamine-pyrrole 2,3-dioxygenase (IDO) and arginase 1 that starve immune cells from essential amino acids (Nduom, Weller and Heimberger, 2015). The brain is especially vulnerable to depletion of tryptophan and arginine, which leads to suppressed T cell proliferation and activation, as these essential amino acids are actively transported across the blood brain barrier and sustained at around 10% of normal plasma levels (Hawkins *et al.*, 2006). In addition, a large percentage of GBM express PD-L1 (61%) albeit at a low level with median percentage expression of 2.77% but although low, the expression of PD-L1 is a significant negative prognostic factor (Nduom *et al.*, 2016). Other than tumour secreted factors, the general microenvironment is a hostile one for T cell recruitment and activation with high levels of hypoxia, acidosis and low nutrient availability also severely hampering effector function of TILs (Wei *et al.*, 2011; Sukumar, Roychoudhuri and Restifo, 2015; Pilon-Thomas *et al.*, 2016).

1.3.2. Current therapies available

There have been a plethora of pre-clinical and clinical studies looking to utilise various forms of immunotherapies for the treatment of GBM. In this section, I will explore them in more detail.

1.3.2.1. Adoptive cell transfer

The main focus of cellular based immunotherapies in GBM have been CAR-T cell therapy. There have been various CAR-T cells trialled, with the most common targets undergoing clinical trials including EGFRvIII (O'Rourke *et al.*, 2017) (NCT01454596, NCT02664363, NCT02209376), interleukin receptor 13Rα2 (IL13Rα2) subtype (Brown *et al.*, 2016) (NCT02208362), epidermal growth factor receptor 2 (HER2) (Ahmed *et al.*, 2015, 2017) (NCT02442297, NCT01109095) and ephrin type-A receptor 2 (EphA2) (NCT02575261).

Whilst there has been some success, notably with IL13Rα2 CAR-T cells (Brown *et al.*, 2016) providing tumour regression and maintenance for over 7 months with repeated infusion into the CSF, the efficacy of such treatments is limited in durability. This is because none of the common tumour associated antigens targeted are present in all GBM tumours and even if present are only expressed in a proportion of the tumour cells (Yan *et al.*, 2009; Montano *et al.*, 2011; Del Vecchio *et al.*, 2013; Ahmed *et al.*, 2015; Felsberg *et al.*, 2017). Even the most common mutation in GBM, the EGFRvIII mutation is only seen in 30% of patients and of those 30% only up 86% of the tumour express the mutation (Wikstrand *et al.*, 1997) meaning tumour escape is therefore inevitable. There are more recent studies looking at bispecific CAR-T cells, which may help address this problem (Hegde *et al.*, 2015; Choi *et al.*, 2019) and it will be interesting to see how they perform in clinical trials.

Other adoptive cell transfer techniques trialled include transfer of TIL. This technique was effective at treatment of metastatic melanoma brain tumours (Hong *et al.*, 2010; Mehta *et al.*, 2018) and was trialled in a pilot study for GBM showing safety in 6 patients and partial response in 3/6 patients (Quattrocchi *et al.*, 1999). However, various technical factors, including difficulty of isolating and expanding TIL from GBM means this approach has not

been adopted more widely. A more controversial therapeutic approach has been the adoptive transfer of CMV specific TIL (Schuessler *et al.*, 2014) based on the finding the CMV was found in GBM as opposed to healthy tissues (Mitchell *et al.*, 2008; Scheurer *et al.*, 2008), a finding which has subsequently been disputed (Priel *et al.*, 2015; Ding *et al.*, 2019). However, a clinical trial to investigate the feasibility and effectiveness of this approach is underway (NCT02661282).

1.3.2.2. Oncolytic viruses

Various types of oncolytic virus have been trialled for the treatment of GBM including herpes simplex virus (HSV), adenovirus, poliovirus, parvovirus, reovirus, measles virus and more recently Zika virus (Kaufman, Kohlhapp and Zloza, 2015). As mentioned earlier, the success of such an approach, regardless of virus used, requires the successful replication of the virus balanced with an active immune response against infected cells. Any shift in this fine balance can lead to either uncontrolled viral infection, or more likely, eradication of the virus and lack of any therapeutic effect.

The first large study to show effectiveness of an oncolytic virus approach was published in 1991 using a modified HSV that specifically targeted rapidly dividing GBM cells *in vitro* and tumour regression *in vivo* (Martuza *et al.*, 1991). This has been followed up with many phase I trials in humans which proved safety (Markert *et al.*, 2000, 2009; Rampling *et al.*, 2000). However, reports looking into their efficacy are still awaited. The added benefit of viral therapy is that it is relatively easy to modify viruses to also include extra transcripts to make infected cells produce immunoinflammatory cytokines and increase the local inflammatory response. The M032 is such an example of a HSV virus that has been

additionally modified to include the human IL12 gene which is currently undergoing a phase I trial (NCT02062827) (Patel *et al.*, 2016).

Adenoviruses have also been studied extensively since it was shown that a modified adenovirus lacking E1B gene is able to infect and kill p53-deficient tumours (Patel *et al.*, 2016). This approach was successfully translated into a clinically viable treatment, ONYX-015, which has been shown to be safe in phase I trials (Chiocca *et al.*, 2004). However, there was no conclusive evidence to show any anti-tumour efficacy and following an unsuccessful phase III trial in head and neck cancer (NCT00006106), development was discontinued. A second generation virus, DNX-2401, has been shown in mouse models to elicit a specific anti-tumoral immune response (Chiocca *et al.*, 2004) and a phase I clinical trial has demonstrated its safety and potential efficacy with 20% of patients surviving for over 3 years of which 3 patients had a 95% reduction in tumour volume (Lang *et al.*, 2018). In addition to their role as oncolytic viruses directly, researchers have utilised adenoviruses as vehicles for gene-mediated cytotoxic immunotherapy. Adenoviral vectors encoding the thymidine kinase gene of the HSV have been used in conjunction with systemic injection of a prodrug such as Ganciclovir. The prodrug is phosphorylated inside infected cells, converting the prodrug into its active form leading to termination of DNA replication and cell death (Chen *et al.*, 1994). This has been tested in phase I trials showing safety and potentially some clinical efficacy (Trask *et al.*, 2000; Chiocca *et al.*, 2011). However, whilst a larger phase III trial did show some improvement in progression free survival (PFS), there was no effect on overall survival (Westphal *et al.*, 2013).

More recently, following an outbreak of the virus in 2017, Zika virus has been explored for the treatment of GBM given its propensity to infect neural precursor cells. So far only

experimental models have been trialled but there is some evidence to support its efficacy in targeting GBM stem cells for therapeutic benefit (Zhu *et al.*, 2017, 2020; Chen *et al.*, 2018) although much more work is required before it can be tested clinically.

1.3.2.3. Vaccination strategies

Similar for other cancer types, there have been many vaccination approaches undertaken in the treatment of GBM.

1.3.2.3.1. Peptide vaccines

Among peptide vaccines, the EGFRvIII targeted vaccinations have received the most attention. The most advanced vaccination against this mutation, Rindopepimut with human GM-CSF adjuvant, has been the target of many in-human studies. Phase I/II trials have shown it is safe and induces an effective immune response against tumours cells expressing the mutation, leading to loss of the mutation in the recurrent tumour (Sampson *et al.*, 2011; Schuster *et al.*, 2015). However, PFS after 5.5 months was only 66% in the largest ACT III and larger phase III study was withdrawn early due to concerns over lack of effect on overall survival (Weller *et al.*, 2017). A similar blinded phase II study ReACT also found no significant difference in PFS at 6 months (28%) compared to Bevacizumab treatment alone (16%) for the treatment of EGFRvIII positive recurrent GBM (Reardon *et al.*, 2020).

Other single targets under investigation include survivin (NCT02455557), Wilm's tumour gene 1 (WT1) (Izumoto *et al.*, 2008; Hashimoto *et al.*, 2015) and isocitrate dehydrogenase

(IDH1) mutation (Schumacher *et al.*, 2014; Platten *et al.*, 2018). Among these, the IDH1 mutation is the most promising as it is known to be a key driver in tumorigenesis, which if present, is expressed by all the cells in the tumour. However, whilst IDH1 mutations are present in over 70% of low-grade tumours, its presence in GBM is much lower (Yan *et al.*, 2009).

One of the pitfalls of a single peptide approach is, similar to mono-specific CAR-T cells, the heterogeneity of expression of mutations leading to tumour escape (Sampson *et al.*, 2011; Del Vecchio *et al.*, 2013). In addition, single peptide vaccinations, such as WT1 which are restricted to a single HLA haplotype (HLA-A24:02 in this case) (Hashimoto *et al.*, 2015), further limits the utility of such vaccinations to a smaller group of patients Like, bi-specific CAR-T cells, this can be overcome by using multiple peptide vaccinations. Such examples include the IMA-950 peptide vaccination, which is an 11-peptide vaccination chosen based on enriched expression in GBM compared to normal brain from mass spectrometry experiments. A phase I/II trial suggested the peptide vaccine is safe and there was a PFS at 6 months of 74% and 31% at 9 months (Rampling *et al.*, 2016). Further early phase trials using IMA-950 adjuvanted with poly-ICLC showed a CD8 T cell response to one or more peptides in 63.2% or 36.8% of patients, respectively, and a median survival of 19 months for the 16 GBM patients in the trial (Migliorini *et al.*, 2019). Other approaches have been to provide personalised vaccinations targeted specifically at neoantigens (Keskin *et al.*, 2018) or a combination of both (Hilf *et al.*, 2018). In the pure neoantigen vaccination trial, the 8 patients enrolled had a median PFS of 7.6 months and overall survival of 16.8 months. Only 3 patients completed the vaccination protocol due to disease progression in the others. Neoantigens-reactive T cells were only detected in the blood of patients who did not receive dexamethasone (Keskin *et al.*, 2018). In a study where a standard TAA

vaccination was first given followed by neoantigen vaccinations, 16 patients had a median PFS of 14.2 months and an OS of 29.0 months. Of the patients who received the vaccinations, 92% were able to generate an MHC class I-restricted T cell reaction to the tumour associated antigens and 80% were able to generate a response to the neoantigens although many of these TILs were still exhausted. This would suggest there is a potential role for mixed tumour associated/neoantigen pooled peptide vaccinations in the treatment of GBM.

1.3.2.3.2. Dendritic Cell vaccines

There have been many DC-based vaccination strategies trialled for GBM. At the most simple level, individual (Sampson *et al.*, 2009) and multiple peptide (Phuphanich *et al.*, 2013) loaded DCs have been trialled in a small numbers of patients with some success. ICT-107 is a DC vaccine loaded with 6 cancer testis and GBM associated antigens (AIM-2, MAGE1, TRP-2, gp100, HER2, and IL13Rα2) and achieved a median PFS of 16.9 months and a median survival of 38.4 months. However, a randomised phase II study showed only a slight 2.2 months PFS advantage and no significant improvement in survival (Wen *et al.*, 2019).

More commonly, tumour cells or cell lysates have been pulsed into DCs. Advantages of this system is that it allows for unbiased loading of the complete spectra of tumour peptides on to all of the relevant MHC class I and II alleles. The most advanced vaccine, DC Vax-L (autologous DCs pulsed with tumour lysate) has progressed to a randomised phase III trial, in which initial reports suggest there may be an improvement in survival on an intention to treat analysis (Liau *et al.*, 2018). However, the reliability of this report is questionable

given it is an interim report of the data with lack of unblinding and detailed analysis of all factors including PFS, which was their started primary end point.

Another potential source of antigens is to selectively use tumour initiating or tumour stem cells. The theory behind this is that by enriching for the tumour stem fraction, the DCs are then loaded with antigens that are present in the populations of cells that give rise to any tumour recurrence in order to focus the immune response against the relevant cells. These techniques are complex and expensive and unlikely to be reproducible on a large scale. However, a phase I/II study utilising autologous tumour stem cell mRNA transfected DCs in 7 patients showed safety and an increased PFS of 23.1 months vs. 7.9 months in a matched historical control.

1.3.2.4. Immune checkpoint inhibitors

Check point inhibitors, as mentioned, have revolutionised the management of some previously untreatable cancers such as metastatic melanoma (Wolchok *et al.*, 2013; Topalian *et al.*, 2014; Larkin *et al.*, 2015; Robert *et al.*, 2015; Schadendorf *et al.*, 2015), non-small cell lung cancer (Borghaei *et al.*, 2015; Brahmer *et al.*, 2015; Garon *et al.*, 2015; Rizvi *et al.*, 2015; Herbst *et al.*, 2016; Reck *et al.*, 2016) and renal carcinoma (Motzer, Escudier, *et al.*, 2015; Motzer, Rini, *et al.*, 2015). GBM has been found to have very high levels of PD-L1 expression on the tumours of up to 88% (Berghoff *et al.*, 2015). Coupled with the knowledge that the majority of TIL in GBM are PD1 positive and exhibit an exhausted phenotype (Berghoff *et al.*, 2015; Woroniecka *et al.*, 2018), there was hope that check point inhibitors would prove to be an effective treatment. There are many clinical trials ongoing (Preusser *et al.*, 2015), but very few have published results. However, the ones

with published results show that response seen in GBM is much more modest than expected (Yarchoan, Hopkins and Jaffee, 2017). The largest study so far, the CheckMate 143 trial, showed that although some patients (3/40) showed partial response and 8/40 had stable disease with checkpoint inhibitor therapy, there was no difference in overall survival in any of the cohorts (Omuro *et al.*, 2017). Proponents of check point inhibitor therapy may argue that treatment of recurrent GBM is very different to targeting primary GBM as patients are often less well and have had immune-ablating therapies such as temozolomide and corticosteroids. The timing of check point inhibitor therapy has also come into question, with some more recent studies suggesting that neo-adjuvant therapy is more effective than adjuvant therapy as it gives pre-existing TILs the chance to become rejuvenated in the presence of cognate antigen, which is not present post-operatively as the tumour has been removed (Cloughesy *et al.*, 2019; Schalper *et al.*, 2019).

Increasing amount of studies have been showing that efficacy of check point inhibition is directly linked to the tumour mutational burden (Yarchoan, Hopkins and Jaffee, 2017) and by extension the neoantigen burden (Mcgranahan *et al.*, 2016; Yarchoan *et al.*, 2017). Unfortunately, GBM is known to be on the low mutational burden side of the spectrum (Alexandrov *et al.*, 2013) and this has been used to explain, at least in part, why GBM do not respond to check point inhibitor therapy as well as would be expected. This is supported by 2 studies where checkpoint inhibitor therapy was successful in treating GBM patients who harboured DNA mismatch repair gene mutations, and a consequent hypermutated phenotype (Johanns *et al.*, 2016; Tabori *et al.*, 2016). Indeed, along this theme, there are ongoing clinical trials investigating the role of radiation therapy in conjunction with checkpoint therapy (Chow *et al.*, 2015) as well as checkpoint inhibitor

therapy for a subset of GBM patients with IDH mutations in which their tumours become secondary hypermutated following temozolomide treatment (NCT02658279).

Studies have shown that TIL in GBM exhibit a severe exhausted phenotype with co-expression of LAG3, TIM3 with PD-1 (Woroniecka *et al.*, 2018) and it maybe that inhibition of just the PD1/PD-L1 axis is insufficient to reverse this exhausted phenotype (Mohme *et al.*, 2018). Indeed, preclinical work suggests that combinations of TIM3 (Kim *et al.*, 2017) or TIGIT (Hung *et al.*, 2018) blockade in addition to anti-PD1 may act synergistically to increase response. Along this line there are clinical trials underway targeting these alternate immune checkpoints either alone or in combination with PD1 inhibition (NCT02658981, NCT02817633).

1.3.2.5. Combination therapies

Given the inherent limitations of each immunotherapeutic strategy, researchers and clinicians are increasingly moving towards combinatorial therapies. Combinations involving checkpoint inhibitor therapies are especially attractive due to their efficacy in other tumours, such as melanoma. Various combinations have been trialled to try and increase their efficacy in GBM including: 1) increasing mutations by radiation therapy (Chow *et al.*, 2015) or following temozolomide treatment (NCT02658279); 2) increasing local inflammatory response and antigen presentation through gene-mediated cytotoxic therapy (Samson *et al.*, 2018; Zadeh *et al.*, 2018); 3) increasing pool of tumour reactive T cells by vaccination (Peters *et al.*, 2019) (NCT04201873) or adoptive cell transfer (NCT03726515) and 4) a combination of the above (NCT03018288). Such strategies are in their infancy and results from this are eagerly awaited.

1.4. Role of epigenetics in cancer

Epigenetics refers to the mechanisms involved in the control of gene expression through means other than that encoded in the genome. The human genome is tightly packed as chromatin in the nucleus. Chromatin itself consists on nucleosomes formed of octamers of histone proteins upon which the DNA is wrapped around. In this state the DNA is inaccessible to RNA polymerases for transcription and the genes are silenced. Histones and DNA itself are capable of undergoing chemical alterations at certain amino acids or nucleotides that will alter their structure and DNA availability for transcription. Through this way, genetically identical cells are able to achieve distinct and diverse phenotypes. Histone acetylation and methylation as well as DNA methylation act as the main epigenetic modifications that control gene expression. It has been found over the last 20-30 years that in addition to mutations causing up/downregulation in gene function, epigenetic factors also play a key role in the aberrant gene expression seen in cancers (Sharma, Kelly and Jones, 2010; Easwaran, Tsai and Baylin, 2014; Kondo *et al.*, 2014; Feinberg *et al.*, 2016).

The most widely studied epigenetic alteration has been DNA methylation due to its stability and the relative ease at which it can be studied (Laird, 2010). It has been shown that epigenetic silencing through DNA methylation plays a key role in the silencing of tumour suppressor genes and can even act as a 'hit' in Knudson's two-hit hypothesis (Jones and Baylin, 2002; Shen and Laird, 2013) and that some of these epigenetic changes are key driver events that are absolutely essential for cancer cell survival (De Carvalho *et al.*, 2012). The importance of these epigenetic changes is such that they are forming key markers in classification of tumours for example, it was first noted in colorectal cancer that there were a subset of tumours that had aberrant hypermethylation of many CpG islands in promoter

regions, analogous to hypermutated phenotypes in patients with DNA mismatch repair gene mutations (Toyota *et al.*, 1999). This hypermethylation pattern has also been found in gliomas associated with IDH1 mutations (Noushmehr *et al.*, 2010) and now forms an independent subclassification of tumours, CpG island methylator phenotype (CIMP), proffering a positive survival advantage.

In fact, other than the CIMP phenotype, there are many other important epigenetic factors identified in gliomas and there are various groups advocating stratification of glioma based purely on DNA methylation states (Capper *et al.*, 2018). A subset of patients' tumours are hypermethylated at the O(6)-Methylguanine-DNA methyltransferase (MGMT) promoter site. MGMT is a suicide DNA repair protein which protect cells from alkylating agents, such as temozolomide. Therefore methylation of MGMT promoter is a positive prognostic factor conferring better survival following alkylating chemotherapy treatment (Gerson, 2004; Hegi *et al.*, 2005; Ma *et al.*, 2009). The role of epigenetic markers is even more pronounced in paediatric gliomas with the H3.K27M mutant a key epigenetic marker in diffuse intrinsic pontine glioma (DIPG) in children (Venneti *et al.*, 2014; Louis *et al.*, 2016). This key epigenetic driver is also the target of many therapies aimed at this surgically inaccessible tumour (Hashizume, 2017; Balakrishnan *et al.*, 2019).

Epigenetic modulators are also being increasingly trialled in the treatment of cancers for varying rationale. Histone deacetylase inhibitors (HDACi) have been shown to cause cell cycle arrest and induce apoptosis, induce differentiation of tumour stem cells, inhibit angiogenesis as well as having some pro-inflammatory responses by increasing PD-L1 expression on tumours and reducing tumour associated macrophages and Tregs and are approved clinically for the treatment of cutaneous T cell lymphomas (Bolden, Peart and

Johnstone, 2006; Youn *et al.*, 2013; Bezecny, 2014; Chueh *et al.*, 2015; Lee *et al.*, 2015; Singh *et al.*, 2015; Grinshtein *et al.*, 2016; Terranova-barberio *et al.*, 2017; Bruyer *et al.*, 2018; Ellmeier and Seiser, 2018). DNA methyltransferase inhibitors (DNMTi) are licensed for the treatment of myelodysplastic disease but have also been shown to be potentially efficacious in the management of colorectal and classical Hodgkin lymphoma in combination with checkpoint inhibitors (Nie *et al.*, 2019; Yu *et al.*, 2019). This class of drugs is especially interesting as they have been shown to be pro-immunogenic for a variety of reasons including: 1) reversing exhaustion/enhancing T cell effector function (Yau *et al.*, 2018); 2) increasing T cell diversity; 3) increasing expression of immunogenic pathways (Konkankit *et al.*, 2011; Chiappinelli *et al.*, 2015; Riccadonna *et al.*, 2016); 4) reducing myeloid derived suppressor cell trafficking to tumour sites (Lu *et al.*, 2020); 5) increasing antigen presentation/co-stimulation related genes on tumours (Zhang *et al.*, 2016; Siebenkäs *et al.*, 2017) and 5) increasing expression of immunogenic antigens such as NY-ESO (Natsume *et al.*, 2008; Konkankit *et al.*, 2011; Everson *et al.*, 2016; Siebenkäs *et al.*, 2017). To date, no one has published any studies investigating the role of epigenetic modulators in the regulation of neoantigen expression.

Given epigenetic silencing plays an important role in the formation and survival of cancer, we hypothesise that there are potential neoantigen encoding mutations in cancers that are not/lowly expressed either because they are passenger mutations or that they have been downregulated through immunoediting. Increasing the expression of these mutations can increase the number of neoantigens expressed by GBM and increase its immunogenicity leading to neoantigen specific killing. This increase in neoantigen expression will hopefully render other immunotherapies such as checkpoint inhibition more efficacious.

1.5. Aims of thesis and outline

In this thesis I aim to address the hypothesis that epigenetic regulation of immunogenic antigens can be manipulated to improve the efficacy of immunotherapy for this otherwise immune 'cold' tumour. To do this, I will explore two broad topics. Firstly, I will investigate the expression of neoantigens and cancer testis antigens in GBM and whether we can increase their expression through modulation of the epigenome. I will then examine whether the increased expression of these immunogenic antigens leads to increased immunogenicity as seen by increased cytotoxic T lymphocyte (CTL) activation and killing. Secondly, I will also look to in-depth characterise the TCR repertoire of TILs in GBM in order to understand which proportion of TILs is actually tumour reactive and if we can enhance the endogenous TIL responses through modulation of the epigenome. The results are set out in the following chapters:

Chapter 3: Decitabine upregulates cancer testis and neoantigens in glioblastoma cell lines.

Chapter 4: Isolating tumour and peptide specific T cells.

Chapter 5: Increased expression of neoantigen and cancer testis antigen leads to increased tumour immunogenicity.

Chapter 6: Single cell analysis of tumour infiltrating lymphocyte repertoire.

2. Materials and Methods

2.1. Media and buffers

Wash buffer: RPMI + 10mM HEPES (Sigma)

Serum free media for primary GBM culture: RHB-A (Takara) + 20ng/mL EGF + 20ng/mL FGF (Peprotech) + 100U/mL penicillin-0.1mg/mL streptomycin (Sigma)

D10: DMEM (Sigma) + 10% foetal calf serum (*v/v*) (FCS, Gibco) + 100U/mL penicillin-0.1mg/mL streptomycin (Sigma)

R10: RPMI (Sigma) + 10% FCS (*v/v*) + 50µM 2-mecaptoethanol + 2mM L-Glutamine + 1x non-essential amino acids + 10mM HEPES + 1mM sodium pyruvate (Gibco) + 100U/mL penicillin-0.1mg/mL streptomycin (Sigma)

RHS5: RPMI (Sigma) + 5% human serum (*v/v*) (pooled serum from 10 different donors obtained from NHS blood and transplant services) + 50µM 2-mecaptoethanol + 2mM L-Glutamine + 1x non-essential amino acids + 10mM HEPES + 1mM sodium pyruvate (Gibco) + 100U/mL penicillin-0.1mg/mL streptomycin (Sigma)

RHS5/IL-2: RHS5 + 500-1000IU IL-2 (produced in house from J588L cell line (Tunnacliffe *et al.*, 1989))

Freezing media: FCS (*v/v*) + 10% DMSO (*v/v*)

FACS buffer: PBS (*v/v*) + 3% FCS (*v/v*) + 1mM ethylenediaminetetraacetic acid (EDTA)

Western blot lysis buffer: 50 mM Tris pH 7.5 + 150 mM NaCl + 1 mM EDTA + 0.5% NP40 (*v/v*) (IGEPAL CA-630) + 1x cOmplete protease inhibitor (Roche)

Western blot Blocking buffer: PBS + 0.1% Tween (*v/v*) + 5% BSA (*w/v*)

2.2. Patients and Human Samples

All human samples were collected through the Oxford Brain Bank, approved by the local research ethics committee (15/SC/0639). Specific informed consent was mandatory for each patient and was obtained before each surgery for patients undergoing multiple operations. All patients were >18 years old and undergoing surgery for a radiologically diagnosed diffuse glioma.

For each patient, fresh tumour samples and 50ml of fresh blood were obtained at the time of surgery. All tumour samples were placed in ice-cold PBS or DMEM (Sigma) during transportation and processed within 2 hours of surgery. Tumour samples were first washed in PBS to remove blood, blood vessels and necrotic tissue were dissected, and the remaining tumours were then mechanically dissociated using a scalpel into small pieces (<2mm in size). The tumour samples were then dissociated enzymatically using a tumour dissociation kit as per manufacturer's instructions (tumour dissociation kit, human, Miltenyi). The single cell suspension was then washed and underwent myelin removal (myelin removal beads, Miltenyi) and CD45 separation (CD45 separation beads, Miltenyi) using MACS column as per manufacturer's instructions. The CD45- fraction was used for establishment of primary cell lines. The CD45- fraction was used for single cell TCR sequencing (scTCRseq) where appropriate. Any remaining samples were frozen in freezing media and stored in liquid nitrogen for future use.

Peripheral blood mononuclear cells (PBMC) were obtained from the fresh blood using established protocols. In short, blood was diluted 1:1 in wash buffer, 30ml of diluted blood was carefully layered on 15ml lymphoprep (Stem cell technologies) and spun for 30minutes at 2000rpmwith minimum acceleration and deceleration. The buffy coat was obtained

using a Pasteur pipette, washed twice in wash buffer at 1500rpm for 15 minutes followed by 1000rpm for 10minutes. Approximately 1 million PBMC were taken for scTCRseq where appropriate and the rest frozen in freezing media for future use.

2.3. Culture of cell lines

2.3.1. Primary GBM cell lines

Primary tumour cell lines were established by seeding either $1x10^6$ or $5x10^5$ fresh CD45- tumour cell digests in $75cm^2$ or $25cm^2$ flasks, respectively. Cells were grown in serum free media. Culture vessels were coated with laminin (Sigma) at 10µg/ml for a minimum of 2hr and subsequently washed with PBS before use. Cells were regularly observed and split 1:3-1:5 using accutase (Biolegend) when confluent. All experiments with primary cell lines were done using early passage cells (passage<15).

2.3.2. Commercial tumour lines

U87MG (donated by Prof. Valentine Macaulay, Department of Oncology, University of Oxford), HEK293T and normal human astrocytes (NHA; donated by Dr Marta Pojo, IPO, Lisbon) were all grown in D10. Jurkat cells were cultured in R10.

2.3.3. Immune cells

Primary immune cells were cultured in a base media of RHS5. For T cell maintenance RHS5/IL-2 was used. Additional cytokines were added to this media, for specific protocols, as stated in the relevant section.

Single cell clonal expansion was performed by sorting single T cells into 96-well tissue culture plates loaded with $1.25x10^5$ irradiated allogeneic feeder cells in 150µL RHS5/IL-2 + 1µg/mL of phytohaemagglutinin (PHA; Remel). Expanding clones were then split as required on reaching confluency and the media turning yellow. Following 2-4 weeks in culture they were then tested by intracellular staining (ICS). For bulk T cell expansion, cells were sorted in bulk and expanded in RHS5/IL-2 + PHA with irradiated feeders at a ratio of 1:200. To prepare irradiated feeder cells, allogeneic PBMC from a minimum of 2 donors were irradiated (30Gy) and pooled in a ratio of 1:1.

2.4. Antibodies

All antibodies and cell staining reagents used in flow cytometry and Western blot experiments are listed in table 2.1.

Table 2.1 Antibodies and staining reagents. All antibody targets are human except when specified otherwise.

Target	Clone	Fluorochrome	Company	product code	Dilution
41BB	4B4-1	APC	Biolegend	309809	1:100
41BB	4B4-1	PerCP-Cy5.5	Biolegend	309813	1:100
41BB	4B4-1	APC-Cy7	Biolegend	309829	1:100
CD107a	H4A3	PerCP-Cy5.5	Biolegend	328616	1:50
CD133	CLONE7	BV421	Biolegend	372807	1:100
CD15	W6D3	BV650	Biolegend	323033	1:100
CD3	HIT1a	PE-Cy7	Biolegend	300316	1:100
CD38	HIT2	APC	Biolegend	303509	1:100
CD38	HIT2	BV711	Biolegend	303527	1:100
CD39	A1	BV421	Biolegend	328213	1:100
CD4	RPA-T4	FITC	BD	555346	1:50
CD4	OKT4	APC	Biolegend	317416	1:100
CD4	OKT4	APC-Cy7	Biolegend	317418	1:100
CD44	BJ18	PerCP-Cy5.5	Biolegend	338819	1:100
CD69	FN50	PE	Biolegend	310906	1:100
CD69	FN50	BV605	Biolegend	310937	1:100

CD8	SK1	BV711	Biolegend	344734	1:100
CD8	SK1	PerCP-Cy5.5	Biolegend	344710	1:100
CD8	SK1	APC-Cy7	Biolegend	344713	1:100
CD8α	HIT8a	PE-Cy7	Biolegend	300914	1:100
CD90	5e10	AF700	Biolegend	328119	1:100
GD2	14G2a	PE	Biolegend	357303	1:100
GD2	14G2a	APC	Biolegend	357305	1:100
GFAP	2E1.E9	AF488	Biolegend	644704	1:100
HER2	24D2	PE-Cy7	Biolegend	324413	1:100
HLA-A2	BB7.2	PE	Biolegend	343305	1:100
HLA-DR	L243	Purified	Biolegend	307648	1:100
HLA-DR	TU36	PE	Biolegend	361605	1:100
TCRβ	T10B9	FITC	BD	555547	1:50
IFN-γ	4s.B3	APC-Cy7	Invitrogen	47-7319-42	1:100
IFN-γ	4s.B3	APC	Biolegend	502512	1:100
IL13RA2	REA308	APC-VIO770	Miltenyi	130-104-555	1:100
IL13RA2	REA308	PE-VIO770	Miltenyi	130-104-671	1:100
MHC class I	W6/32	PE	Biolegend	311405	1:100
MHC class I	W6/32	Purified	Biolegend	311428	10µg/mL
MHC Class II	Tu39	Purified	Biolegend	361702	20µg/mL
MHC Class II	Tu39	FITC	Biolegend	361705	1:100
Mouse TCRβ	H57-597	APC-Cy7	Biolegend	109220	1:100
MR1	26.5	Purified	Biolegend	361109	20µg/mL
MR1	26.5	APC	Biolegend	361107	1:100
MYRF	Polyclonal	Purified	Merck	ABN45	1:1000
NESTIN	196908	PE	R&D Systems	IC1259P	1:100
NG2	LHM-2	APC	Biolegend	FAB2585A	1:100
NY-ESO	E978	Purified	Sigma	N2038	1:1000
PD1	eBioJ105	PE-Cy7	eBioscience	25-2799-41	1:100
SLC6A12	Polyclonal	Purified	Life Technologies	PA524005	1:1000
SOX2	REA320	APC	Miltenyi	130-104-995	1:100
Streptavidin		PE	Biolegend	12-4317-87	
Streptavidin		APC	Biolegend	17-4317-82	
TNF-α	MAb11	PE	Biolegend	502909	1:100
Zombie Aqua			Biolegend	77143	1:200

2.5. Tetramers

UV cleavable HLA-A2 tetramers were produced using established protocols (Rodenko *et al.*, 2006). In short, HLA-A2 heavy chain and B2M were refolded in the presence of a

photolabile peptide (KILGFVFJV, Pepscan) to create HLA-A2 monomers. These were purified on fast protein liquid chromatography (FPLC), biotinylated and stored at -80°C until use. To load specific peptides, 10µL of monomer at 200µg/mL and 10µL of peptide at 100µM were mixed and incubated under 366nm light for 1 hour at 4°C. The suspension was then spun at 2250xg for 10minutes and 15µL the supernatant recovered. 2µL of streptavadin conjugated fluorochromes (0.5µg/µg monomer, eBiosciences) plus 10µL of milk at 10% in PBS (w/v) (powder milk, which company?) were then added to the supernatant and incubated at 4°C for at least 1 hour. 10µL containing 2.5uM D-biotin and 0.04% (w/v) sodium azide were then added and incubated for at least 30 minutes before use. All tetramers were kept at 4°C and used within 6 months.

2.6. Peptides

Predicted neoantigen peptides were brought from Pepscan (Netherlands) and Genecust (France) at crude purity and resuspended in DMSO to 10mg/mL stock and stored at -80°C. A list of peptides bought can be found in the appendix.

2.7. Flow Cytometry

All staining steps were done in a dark tissue culture hood. Flow cytometry analysis was acquired using a Fortessa x20, x50 (BD) or Attune NxT (Life technologies) flow cytometers. Data was analysed using FlowJo.

2.7.1. Cell surface staining

Cells were washed in PBS and resuspended in 20uL of live/dead marker zombie aqua (1:200 in PBS, Biolegend) for 15 minutes at room temperature. 20µL of the relevant antibody mix diluted, as per table 2.1, in FACS buffer was then added and incubated for a further 25-30 minutes on ice. Samples were then washed with PBS and resuspended in 200µL FACS buffer before analysed on a flow cytometer.

2.7.2. Intracellular staining

Cells were stained for cell surface markers as described above. Cells were then washed and resuspended in 50-100µL of 1x IC Fixation Buffer (eBioscience) and incubated at 4°C for a minimum of 30 minutes. Cells were subsequently washed twice in 1x permeabilization buffer (eBioscience) and resuspended in 20µL antibody mix diluted, as per table 2.1, in 1x permeabilization buffer and incubated for 30 minutes on ice. Cells were then washed in 1x permeabilization buffer once followed by before being resuspended in 200µL FACS buffer.

2.7.3. Tetramer staining

Cells were washed in PBS and resuspended in 100µL of PBS. 2µL of the tetramer was added to the suspension and incubated at 37°C for 20-25 minutes. Cells were washed in PBS and further cell surface staining was performed as described above.

2.7.4. Cell sorting

Cells surface staining was performed as described above and cells were sorted into either 2ml tubes, 96-well tissue culture and PCR plates or 384-well PCR plates. Cell sorting was performed using either Fusion 2, Aria III (BD) or SH800 (Sony) cell sorters.

2.8. Decitabine treatment of cell lines

Cells were passaged and re-plated the day before treatment as per standard practice and cultured overnight. Cells were treated with the required amount of decitabine every 24h, for a total of 48h. Due to its poor stability, decitabine was diluted in media immediately before each application using single-use aliquots of 10mM decitabine stock stored at -80°C. Cells were then cultured with media without decitabine for a further 3 days for transcriptomic analysis or a further 5 days for proteomic and functional assays. A paired sample was treated in an identical way without decitabine as an untreated control.

For testing of cell growth and viability, $2x10^4$ U87MG cells were plated in a T25 flask and treated with/without 1µM DAC in parallel. Cells were dissociated on days 1,3,6 and 9 and cell number and viability measured using the trypan blue using a Countess™ II FL Automated Cell Counter (ThermoFischer Scientific).

2.9. Generation of peptide and tumour specific T cells

2.9.1. Peptide stimulation of patient PBMC

Peptide stimulation was performed using a standard protocol in our laboratory (Chen *et al.*, 2015). Patient PBMC were resuspended at $1.5x10^7$ cells/mL in 200µL RHS5 + 10ng/mL

IL-7 (Peprotech) in a 48-well plate. 1.5mM pooled peptides were added to the PBMC and incubated at 37°C for 30 minutes. A further 800µL RHS5 + 10µg/mL IL-7 was added to dilute the peptide pool to 30µM in 1mL of culture. After 3 days, IL-2 was added to the media to a final concentration of 1000IU/mL. PBMC were monitored and subsequently split as required over the next 2 weeks. Cells were then analysed for peptide specificity by tetramer staining and sorted in bulk gated on CD3+/CD8+/tetramer+ cells for expansion.

2.9.2. In vitro priming of healthy donors

PBMC were isolated from HLA-A2 healthy donors and separated by CD14 separation beads (Miltenyi). CD14+ cells were used to produce monocyte derived mature dendritic cells (moDCs): CD14+ cells were cultured at $3x10^5$ cells/mL in R10 + 1000IU IL-4 (produced in house from J588L cell line (Tunnacliffe *et al.*, 1989)) + 50ng/mL human GMCSF (Peprotech) for 4 days before maturation with 2ng/mL TNFα (Peprotech) and 10ng/mL lipopolysaccharide (LPS Salmonella abortus aequi, Sigma) for 24 hours. The moDCs were then split evenly and pulsed with 1µM of each individual peptide separately. These cells were then washed twice in PBS and pooled together to create patient specific pools. The peptide pulsed moDCs pools were then mixed with the CD14- fraction at a ratio of 1:10 ($6x10^5$ moDCs: $6x10^6$ CD14- cells in 2mL in one well of a 24-well plate) in RHS5 + 10ng/mL IL-7 + 50pg/mL IL-12 (Peprotech) for 10-14 days with media changed on day 7. The cells were then re-stimulated on day 10-14 with peptide pulsed moDCs at a ratio of 1:5 ($6x10^5$ moDCs: $3x10^6$ CD14- cells in 2mL in a 24-well plate) in RHS5/IL-2. These cells were then cultured for a further 2 weeks, splitting as required. Cells were analysed for peptide specificity by tetramer staining and sorted in bulk gated on CD3+/CD8+/tetramer+.

2.9.3. Mixed tumour lymphocyte culture (MLTC)

Patient PBMC were co-cultured with irradiated autologous primary tumour cell line (30Gy) in RHS5 + 10ng/mL IL-7 + 50pg/mL IL-12 at a ratio of 1:5 ($6x10^5$ PBMC : $3x10^6$ tumour cells in 2mL in a 24-well plate), changing half the media on day 7. The cells were then re-stimulated with further irradiated tumour cells on day 10-14 at a ratio of 1:5 in RHS5/IL-2. The cells were then split as required for 14 days before testing for tumour reactivity by ICS. Further stimulation, to increase the proportion of tumour reactive T cells if required, was performed with irradiated tumour cells at a ratio of 1:5 in RHS5/IL-2. Cells were single cell sorted using both IFN-γ and TNF-α capture assays (Miltenyi) gated on either CD3+/IFN-γ+ positive , CD3/TNF-α positive or CD3+/IFN-γ+/TNF-α+ for both scTCRseq and clonal expansion.

2.9.4. T cell receptor cloning

TCRs of interest were cloned into a modified pHR-SIN plasmid. The TCRα and TCRβ VDJ regions were amplified by polymerase chain reaction (PCR) using specific primers (see appendix) and DNA generated during TCR sequencing library preparation (see 1.14.4). These PCR products were then cleaned using magnetic beads (AMPure XP, Beckman Coulter) and cloned in the plasmid using HiFi DNA assembly cloning kit (NEB). Competent 5-alpha bacteria (NEB) were transformed and grown on ampicillin (100µg/mL, Sigma) infused agar plates. 1-3 colonies were picked and expanded in ampicillin containing LB broth (Sigma) overnight. Plasmid DNA was isolated using QIAprep spin miniprep kit (Qiagen) as per manufacturer's instructions. DNA sequences were confirmed through Sanger sequencing (Source Bioscience).

2.9.5. Lentivirus production

Lentivirus were produced using HEK 293T cells, pMDG-VSVG plasmid which encodes the envelop viral protein and pCMV-dR8.91 packaging plasmid containing the gag/pol and polymerase genes. HEK 293T cells were seeded in 6-well plates at $1x10^6$ million cells per well in 2mL D10 and cultured overnight. On the next day, 1µg of TCR plasmid was mixed with 0.5µg pCMV-dR8.91, 0.25µg pMDG-VSVG, and 4.5µl TurboFectin (Origene) in 100µl of serum free media. This mix was incubated for 30 minutes at room temperature and subsequently added to a well containing HEK 293T cells. Viral supernatants were carefully collected 48h later, centrifuged at 2000rpm for 2 minutes to remove any cells and stored in the fridge for further use.

2.9.6. TCR transduction

Primary CD8 T cells were activated by mixing $1x10^6$ million cells with $1x10^6$ million CD3/CD28 Dynabeads (Thermo Fisher) in 2mL of RHS5/IL-2 supplemented with 10ng/mL IL-15 and cultured in one well of a 24-well plate for 2 days. 48-well plates were treated with 250µL retronectin (Takara) solution at 25µg/mL for a minimum of 16 hours and blocked with 2% BSA in PBS for 15 minutes before use. Lentivirus supernatant was applied into a retronectin-treated well and centrifuged at 2000xg, for 1 hour 30 minutes. The supernatant was discarded and $2.5x10^5$ T cells resuspended in 1mL RHS5/IL-2 supplemented with 10ng/mL IL-15 were added to the well and cultured for 3 days.

TCR transduction into Jurkat cells was simply performed by mixing 600µL of lentivirus supernatant with $5x10^5$ Jurkat cells resuspended in 600µL of R10. Successful transduction was confirmed through mouse TCRβ staining analysed by flow cytometry. Purification was

performed when the transduction efficiency was below 75% by flow cytometry sorting mouse TCRβ+ cells.

2.10. Generation of NFAT-GFP reporter cell lines

Jurkat cells that were endogenous TCRα and TCRβ knock out (KO) and either had expression of endogenous CD4 (J4) or were CD4 KO/and CD8α/CD8β knock in (J8) were kindly donated by Simon Davis group at the Human Immunology Unit, University Oxford. The J4 and J8 were then transduced with green fluorescent protein (GFP) under the control of a NFAT-linked promoter obtained through Addgene (pSIRV-NFAT-eGFP from Peter Steinberger; Addgene plasmid #118031). TCRs of interest could then be transduced into these cells as previously described.

2.11. Functional T cell and Jurkat assays

2.11.1. Cytokine staining

T cells were washed and resuspended at $1x10^5$cells/150µL RHS5 in a 96-well round bottom plate overnight. Tumour cells were resuspended at $5x10^4$ cells/100µL RHS5 in a 96-well round bottom plate overnight. If relevant, tumour cells were pulsed with 1µM peptide (or less for peptide titration assays) for 1 hour at 37°C and washed twice in PBS. The following day, tumour cells were resuspended in 50µL RHS5, and if required were further incubated for 1 hour with blocking antibodies (W6/32 at 20µg/mL, Tu39/L243/26.5 at 40µg/mL). T cells were washed and resuspended in 50µL RHS5 + 0.5µL CD107a antibody (Biolegend) and mixed with the tumour cells and incubated at 37°C for 1 hour. For the positive control,

cells were pulsed with 1x cell stimulation cocktail (eBioscience). 1x Protein transport inhibitor cocktail (eBioscience) was then added and the cells incubated for a further 4 hours at 37°C. Cells were then washed in PBS and stained for extracellular markers and intracellular cytokines IFN-γ and TNF-α as previously described.

2.11.2. Jurkat activation assay

Tumour cells were resuspended at $5x10^4$ cells/100µL RHS5 and pulsed with 1µM peptide if required. Cells were then washed and resuspended in 50µL R10, plus blocking antibody, if required, and incubated for 1 hour at 37°C. $1x10^5$/50µL R10 Jurkat cells were then co-cultured overnight (16-18h). Cells were then washed and stained with live/dead marker, mouse TCRβ, and CD69 as previously described

2.11.3. LDH release assay

LDH release assay was performed using the Cyto-tox nonradioactive cytotoxicity assay (Promega) as per manufacturer's instructions. In brief, $4x10^4$ primary cells or $2x10^4$ U87MG were plated in 100µL RHS5 in a 96-well round bottom plate. Cells, when required, were pulsed with 1µM peptide for 1 hour at 37°C. Cells were then washed twice with PBS and resuspended in 50µM RHS5, plus blocking antibody, where appropriate, for 1 hour. T cells were washed and suspended in RHS5 without IL-2. 50µL of T cells were added to the tumour cells in varying effector: target ratios (E:T; 2:1, 1:1, 1:2 and 1:4) and incubated overnight (16 hours). 1x Lysis buffer was added to the 100% cell death control wells 45 minutes before supernatant harvesting. Cells were centrifuged at 1500rpm for 3 minutes and 50µL of the supernatant was transferred to a fresh 96-well flat bottom plate. 50µL of

substrate mix was added and incubated at room temperature in the dark for 30 minutes. 50µL of stop buffer was added, any large bubbles popped, and emission at 590nm was read on an iMark microplate reader (Bio-Rad). Specific T cell killing was calculated using the following formula:

$$Specific\ Lysis = \frac{Experimental\ readout - T\ cell\ only - tumour\ cell\ only}{tumour\ cell + lysis\ buffer - tumour\ cell\ only}$$

2.12. Western Blotting

Cells were harvested and resuspended in lysis buffer at a concentration of $1x10^8$mL and incubated on ice for a minimum of 30 minutes. The lysate was centrifuged at 10,000 rpm for 10 minutes at 4°C and the supernatant taken and stored at -20°C until further use. Protein quantification was performed using the Pierce BCA protein assay (Thermo Fisher), following manufacturer's instructions. The maximum amount of protein for the lowest concentrated sample was used for all samples, generally between 25ng – 50ng. Lysates were reduced at 95°C for 5 minutes using NuPAGE™ LDS Sample Buffer and NuPAGE™ Sample Reducing Agent (Invitrogen) before running on 4-12% Bis/Tris gels (LifeTechnologies). Gels were then blotted on to PVDF membranes (Bio-Rad) using the Trans-Blot Turbo (Bio-Rad) as per manufacturer's instructions. Membranes were then blocked using blocking buffer for 1-2 hours on a shaker before incubation with the primary antibodies overnight at 4°C. Membranes were washed with wash buffer and stained with secondary fluorescently conjugated antibodies (Li-Cor) for 1 hour at room temperature. Membranes were further washed with wash buffer and dried in the dark before imaging and analysis using Odyssey (Li-Cor).

2.13. Sequencing

2.13.1. Whole exome sequencing (WES) and mutation calling

Genomic DNA was extracted from primary tumour samples, cell lines and PBMC (germ line) using DNEasy blood and tissue kit (Qiagen) as per manufacturer's instructions. WES was performed by Novogene Co. Ltd using SureSelect Human All Exon V6 (Agilent) and Next Ultra DNA Library Prep Kit (NEB). The library was sequenced using the HiSeq platform (Illumina), utilising a paired-end 150 base pairs sequencing strategy. Sequencing was performed at a depth of 50X for PBMC and primary cell lines and 150X for tumour samples.

WES reads were processed, mapped and had somatic variants called using two concurrent workflows. In one workflow, reads were trimmed using Trim Galore (Babraham Bioinformatics) v0.4.1 (utilising Cutadapt version (Martin, 2011) 1.2.1 and FastQC (Babraham Bioinformatics) v0.10.1) and mapped against Homo sapiens genome version hg38 using Bowtie2 (Martin, 2011) v2.2.5. Duplicate reads in mapped files were detected and removed using Picard MarkDuplicates (Broad Institute) v2.3.0 and the resulting mapped sequences were sorted and indexed using Samtools (Li *et al.*, 2009; Li, 2011) v1.6. The other workflow used was GATK4 best practices. Finally, somatic variants were called using Strelka2 (Kim *et al.*, 2018) and GATK's Mutect2 (Broad institute) at the end of each respective workflow. For a final mutation list, the union of somatic variants called from either workflow with a variant allele frequency >5% in the tumour samples and <5% in the PBMC were considered for neoantigen prediction.

2.13.2. RNA sequencing

Cell lines were treated with decitabine as previously described, in three independent experiments. Untreated controls were prepared in parallel. Cells were harvested on day 5 and RNA was extracted using RNEasy kit (Qiagen) as per manufacturer's instructions. 1µg RNA was sent for library preparation and sequencing by Novogene Co. Ltd. Library preparation was performed using Next® Ultra RNA Library Prep Kit for Illumina® (NEB) and sequenced using the NovaSeq platform (Illumina) with a paired-end 150 base pair sequencing strategy.

RNA-seq reads were processed using Trim Galore, Picard MarkDuplicates and Samtools and were mapped against Homo sapiens genome version hg38 using STAR (Dobin *et al.*, 2013) v2.5.3a. The featureCounts tool from the Subread package v1.6.2 was used to quantify reads to generate counts per gene which were then transformed into transcripts per million (TPM) values using an in-house python script. Differential gene expression analysis was performed using DEseq2 (Love, Huber and Anders, 2014). Gene set enrichment analysis (GSEA) was performed using clusterProfiler (Yu *et al.*, 2012) using mSigDB curated gene sets (Subramanian *et al.*, 2005; Liberzon *et al.*, 2011, 2015). Results were filtered with adjusted $p \leq 0.05$. The top 10 gene sets were selected for display using Normalised Enrichment Value (NES) metric.

2.13.3. Methylation sequencing

Global methylation analysis was performed by Diagenode using Infinium methylation EPIC BeadChip Kit (Illumina). Analysis was performed using the ChAMP Bioconductor package (Aryee *et al.*, 2014; Morris *et al.*, 2014). Probes were removed if their detection P-value

exceeded 0.01, and the β-values were normalised by peak-based correction (Dedeurwaerder *et al.*, 2011). The change in methylation between conditions was evaluated in a pairwise fashion. Differentially methylated probes were defined as a probe with an adjusted p-value <0.05 and a differentially methylated gene was defined as a gene with ≥1 differentially methylated probes.

For targeted bisulphite sequencing, 500ng of genomic DNA was bisulphite converted using the Zymo EZ DNA Methylation-Lightning Kit (Cambridge Bioscience) and amplified using the PyroMark PCR kit (Qiagen) according to the manufacturer's recommendations. The PCR primers used to amplify the loci of interests were designed using methprimer (Li and Dahiya, 2002) and contained Illumina adaptors and indexes for library barcoding. The PCR products were purified using the Multiscreen-PCR$_{\mu 96}$ Filter plates (Millipore), quantified by fluorescence using the high sensitivity Quant-iT™ dsDNA Assay Kit (Thermo Fisher Scientific) and equally pooled for each sample. The resultant reads were separated by gene according to primer sequence then corrected for sequencing errors, based on the expected sequence from the GRCh37 reference genome. A maximum of three indels of up to three nucleotides each were corrected if they immediately followed a repetitive region (≥5 nucleotides in which ≥80% of positions were the same base) and did not contain a CpG site. CpG sites, point mutations and more erroneous reads were not changed to avoid false corrections. Bismark was used to extract methylation data (Krueger and Andrews, 2011) and the data are displayed in graphs based on Methylation plotter (Mallona, Díez-Villanueva and Peinado, 2014).

2.13.4. TCR sequencing

TCR sequencing was performed using a modified Smartseq 2 protocol. All primers were bought from IDT; primer sequences and cycling conditions can be found in the appendix. For scTCRseq individual cells were sorted in each well, whereas for mini-bulk sequencing 50 cells were sorted per well. Cells were sorted into either 384-well (single cell) or 96-well (single cell or mini bulk) PCR plates directly into lysis buffer (0.8% triton + RNAse inhibitor (Takara); oligoDT, TCRα/β specific primers (IDT) + dNTP (NEB)). Cells were then snap frozen, stored at -80°C, and processed within 1 month of sorting.

Reverse transcription (RT) and template switching were performed using SmartScribe reverse transcriptase (Takara) and an oligonucleotide containing 3 RNA G bases at the 3' end. On the same day, total cDNA was PCR amplified using SeqAmp kit (Takara) and then snap frozen and stored at -80°C.

The amplification product was then diluted 1:10 and any 96-well plates were miniaturised into 384-well plates. TCR α/β DNA was specifically amplified in two subsequent rounds of nested PCR. Well indexing was performed during the first PCR using 384 different indexed primers that differ in at least 2 bases. 1st PCR products were pooled and cleaned using 0.9x magnetic beads (AMPure XP, Beckman Coulter) A final PCR was performed to add Illumina adaptors and Nextera indexes to identify each PCR plate. Products from each plate were quantified using TapeStation DNA ScreenTape (Agilent), pooled in a 1:1 ratio and cleaned using magnetic beads. The final library was quantified using Qbit high sensitivity DNA quantification kit on Qubit 4 Fluorometer (Thermo Scientific). Sequencing was performed using the Miseq Reagent Kit V2 300 cycle in the MiSeq platform (Illumina). All PCR reactions were performed using ProFlex PCR system (Thermo Scientific) or Alpha Cycler 4 (PCRmax).

FASTQ files for each plate were obtained from BaseSpace (Illumina). Each FASTQ file was demultiplexed for each single cell, and TCR chain by exact barcode match. TCR sequences were analysed using MiXCR (Bolotin *et al.*, 2015) and post analysis were performed using VDJtools (Shugay *et al.*, 2015).

2.13.5. HLA haplotyping

HLA haplotyping was performed by the sequencing facility at the Weatherall Institute for Molecular Medicine, University of Oxford.

2.14. Neoantigen Prediction

Neoantigen prediction was performed by an in-house re-developed version of MuPeXI (A.-M. Bjerregaard *et al.*, 2017) codenamed TUNAPASTA v0.5, which follows much of the same rationale. Additional features incorporated into TUNAPASTA include migration on to Python 3.5.3, ability to take into account the mutant allele frequency and compatibility with Strelka2 (Kim *et al.*, 2018). Patient mutations, HLA-types and TPM expression data are input and processed to determine a ranked list of neoantigens. TUNAPASTA relies on the Ensembl Variant Effect Predictor (VEP) (Jurtz *et al.*, 2017) v92.1, NetMHCpan (Jurtz *et al.*, 2017) v4.0 and Python v3.

2.15. Immunopeptidomics

Approximately 0.6×10^8 or 1.6×10^9 cells were pelleted and 0.5 ml ice cold Igepal buffer (1% IGEPAL CA-630, 100 mM Tris pH8.0, 300 mM NaCl and 1 tablet cOmplete Protease

Inhibitor Cocktail EDTA-free (Roche) per 10 ml buffer) was added per 0.5–1 × 10^8 cells. Cells were then lysed by mixing for 30 min at 4°C in a circular rotator. Lysates were cleared by sequential centrifugation steps at 500 × g for 10 min, then >20,000 × g for 45 min at 4°C. Lysates were diluted to a maximum concentration of 0.5% Igepal with 50 mM Tris. Peptide–MHC class I complexes were extracted by incubation of lysate with 1 mg/10^8 cells of pan MHC class-I antibody (W6/32, HB-95, ATCC) previously cross linked using 40 mM DMP (pH 9) to Protein A Sepharose beads for 30 minutes at room temperature, and incubated overnight at 4°C on a slow speed rotator. The lysate was flowed through a pre-washed chromatography column, then the beads were consecutively washed with a minimum of 10 column volumes of each wash buffer 1 (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA), wash buffer 2 (50 mM Tris pH 8.0, 150 mM NaCl), wash buffer 3 (50 mM Tris pH 8.0, 450 mM NaCl), wash buffer 4 (50 mM Tris pH 8.0). Peptide–MHC complexes were eluted by addition of 5 column volumes of 10% acetic acid and collected in 2ml low-bind centrifuge tubes, then dried in a vacuum drier. Next, peptides were collected into one single tube and re-suspended in 120 µl loading buffer.

RP-HPLC (Reversed-phase high performance liquid chromatography) was used to separate peptides from HLA complexes, α chain and β2-microglobulin. Briefly, samples were injected directly onto column (4.6 x 50 mm ProSwift RP-1S, ThermoFisher Scientific) and eluted during a 10 minutes gradient of 2 to 35% buffer B (0.1% TFA in acetonitrile) into buffer A (0.1 TFA in water) at a flow rate 1000 µl/min using Ultimate 3000 HPLC system (ThermoFisher Scientific). β2-microglobulin-free odd and even fractions were collected into one final odd and one final even fraction. Samples were dried in a vacuum drier, re-suspended in 20 ml loading buffer (0.1% TFA, 1% ACN) and stored at −80 °C until MS analysis.

Peptides were analysed by nano-liquid chromatography–tandem mass spectrometry (nano-LC-MS/MS) using an Ultimate 3000 RSLCnano System (PepMap C18 column, 2 µm particle size, 75 µm × 50 cm; Thermo Scientific) coupled with a Q Exactive HF-X (Thermo Scientific). A 60-min linear gradient from 3% to 25% acetonitrile in 5% DMSO/0.1% formic acid at a flow rate of 250 nl/min was used for peptide elution. MS detection was performed with a resolution of 120,000 for full MS (320-1600m/z scan range) at an AGC target of 300,000, and precursor ions were selected with a 1.6 amu isolation width during a 2.3 second cycle time. MS2 resolution has been set at 60,000 at an AGC target of 50,000 and used high-energy collisional dissociation (HCD) for the fragmentation of precursor ions (25 collision energy for peptides with a charge state of 2-4). Peaks X (Bioinformatics Solutions) was used to analyse MS data by matching the Spectra to a bespoke, patient specific database containing protein variants called from WES datasets (648 entries) and SwissProt reviewed Homo sapiens only database with 20,368 entries (total 21,016 entries). Peaks score cut-off of -10lgP=15 was applied to all samples, and false discovery rate of reported peptide sequences ranged between 2.1 and 5.7 % (average 3.9%).

2.16. Real Time quantitative PCR (RTqPCR)

Cells were treated with/without decitabine as previously described. RNA was extracted using RNEasy kit (Qiagen) and RT was performed using RETROscript (Invitrogen) or Lunascript (NEB). Taqman probes (Life Technologies; see appendix) with either 20ng (96-well plate) or 10ng (384-well plate) of cDNA and the Taqman Fast Advanced Master mix (Life Technologies) were used for the assay. RTqPCR was performed using the QuantStudio 7 (Life Technologies). All analysis was performed using ΔΔCT for a CT value of 0.02.

2.17. Statistical analysis

Statistical analysis for function assays and qPCR was performed using Prism 8 (GraphPad) using two-tailed paired student t test or 2-way analysis of variance (ANOVA) with Sidak's multiple comparisons test as stated in the results. Results are presented as mean with standard errors. P values: * $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$, **** $p \leq 0.00001$.

DEseq2, clusterProfiler and ChMAP were utilised mostly with default conditions, which includes P values adjusted using Benjamini Hochberg multiple tests corrections.

3. Decitabine increases cancer testis antigen and neoantigen expression in glioblastoma cell lines

3.1. Introduction

Check point inhibitor therapy has revolutionised the management of some previously untreatable cancers such as metastatic melanoma (Wolchok *et al.*, 2013; Topalian *et al.*, 2014; Larkin *et al.*, 2015; Robert *et al.*, 2015; Schadendorf *et al.*, 2015), non-small cell lung cancer (Borghaei *et al.*, 2015; Brahmer *et al.*, 2015; Garon *et al.*, 2015; Rizvi *et al.*, 2015; Herbst *et al.*, 2016; Reck *et al.*, 2016) and renal carcinoma (Motzer, Escudier, *et al.*, 2015; Motzer, Rini, *et al.*, 2015). However, their effect in GBM is much more modest (Omuro *et al.*, 2017). As their use has become more widespread, it is now becoming increasingly apparent that checkpoint inhibitor efficacy is directly linked to the tumour mutational burden (Yarchoan, Hopkins and Jaffee, 2017) and neoantigen burden (Mcgranahan *et al.*, 2016; Yarchoan *et al.*, 2017). GBM does not harbour a high tumour mutational burden (Alexandrov *et al.*, 2013; Schumacher and Schreiber, 2015), which may in part explain the poor response rate to such immunotherapies.

We hypothesise that there are potential neoantigen encoding mutations present in GBM that are silenced or lowly expressed. This could either be because it is a passenger mutation in a gene that is not normally expressed in glial cells or possibly due to an active tumour immune escape mechanism. The latter hypothesis is supported by studies in lung cancer which show that patients are less likely to express clonal neoantigens in tumours with high immune infiltrate (Rosenthal *et al.*, 2019). In addition, there is evidence to show that DNA silencing is associated with promoter hypermethylation in cancers (Herman and Baylin, 2003). We further hypothesise that if we could increase expression of these putative

neoantigens then we could enhance the immunogenicity of this otherwise immunologically 'cold' tumour. This could lead to increased immune mediated killing of the tumour directly, but could also synergise with other existing immunotherapies, such as checkpoint inhibition.

To test these hypotheses, we initially interrogated the cancer genome atlas (TCGA) database to explore mutation expression and methylation patterns in the glioblastoma cohort. We then approached this question experimentally, starting with the U87MG commercial cell line before moving on to patient samples and primary cell lines.

To modulate the epigenetic landscape of the cell lines, we used the DNMTi decitabine (DAC). DAC is a cytidine analogue, which incorporates into the genome instead of cytidine during DNA replication. When DNA methyltransferases bind to CpG sites to remethylate the cytidine, DAC occupies their catalytic site blocking the enzymatic activity of DNMTs. Therefore, over many cell cycles there is gradual hypomethylation of the genome (Hagemann *et al.*, 2011; Navada *et al.*, 2014). The benefits of using DAC are threefold: 1) it is licensed clinically for the treatment of myelodysplastic disease (Navada *et al.*, 2014) making any translation into clinical use easier; 2) it has been shown to be pro-immunogenic by increasing expression of antigen presentation machinery, interferon stimulated genes and certain specific cancer testis antigens on tumour cells (Christman, 2002; Hagemann *et al.*, 2011; Gnyszka, Jastrzębski and Sylwia, 2013; Héninger, Krueger and Lang, 2015; Terranova-Barberio, Thomas and Munster, 2016), including gliomas (Natsume *et al.*, 2008; Konkankit *et al.*, 2011; Everson *et al.*, 2016; Riccadonna *et al.*, 2016) as well as increasing the T cell repertoire and reversing T cell exhaustion (Nie *et al.*, 2016; Yau *et al.*, 2018); and 3) there is evidence that combinatorial DAC/checkpoint inhibitor therapy may be beneficial

in certain tumours (Nie *et al.*, 2019; Yu *et al.*, 2019). However, to the best of my knowledge, there have been no reports investigating the role of DAC in modulating expression of neoantigens or the full spectrum of cancer testis antigens.

In this chapter, I describe our analysis of the expression of potential neoantigens and CTA, initially from TCGA and experimentally using the established U87MG cell line and primary cell lines derived from patient samples. I then present data showing the effect of DAC treatment on their expression.

3.2. Results

3.2.1. Neoantigens are silenced by methylation in The Cancer Genome Atlas

We first interrogated the data available on the Cancer Genome Atlas (TCGA, NCI). There were 116 GBM patients with mutation, transcription, methylation and survival data available for which there were >10 and $\leq$200 mutations. Analysis of this cohort of patients shows that there is a trend towards a positive correlation between number of non-silent mutations and survival (figure 3.1A). In addition, by splitting the cohort into thirds, there is a significant improvement in median survival in patients in the upper third of mutations compared to those in the lower third (313 days vs 434 days; figure 3.1B). If expression of these mutations is also accounted for, then there is a greater correlation between number of expressed mutations (FKPM$\geq$1) and survival, which does reach significance (figure 3.1C). Again, patients in the upper third of expressed mutations have a significantly increased median survival (333 days vs 434 days; figure 3.1D).

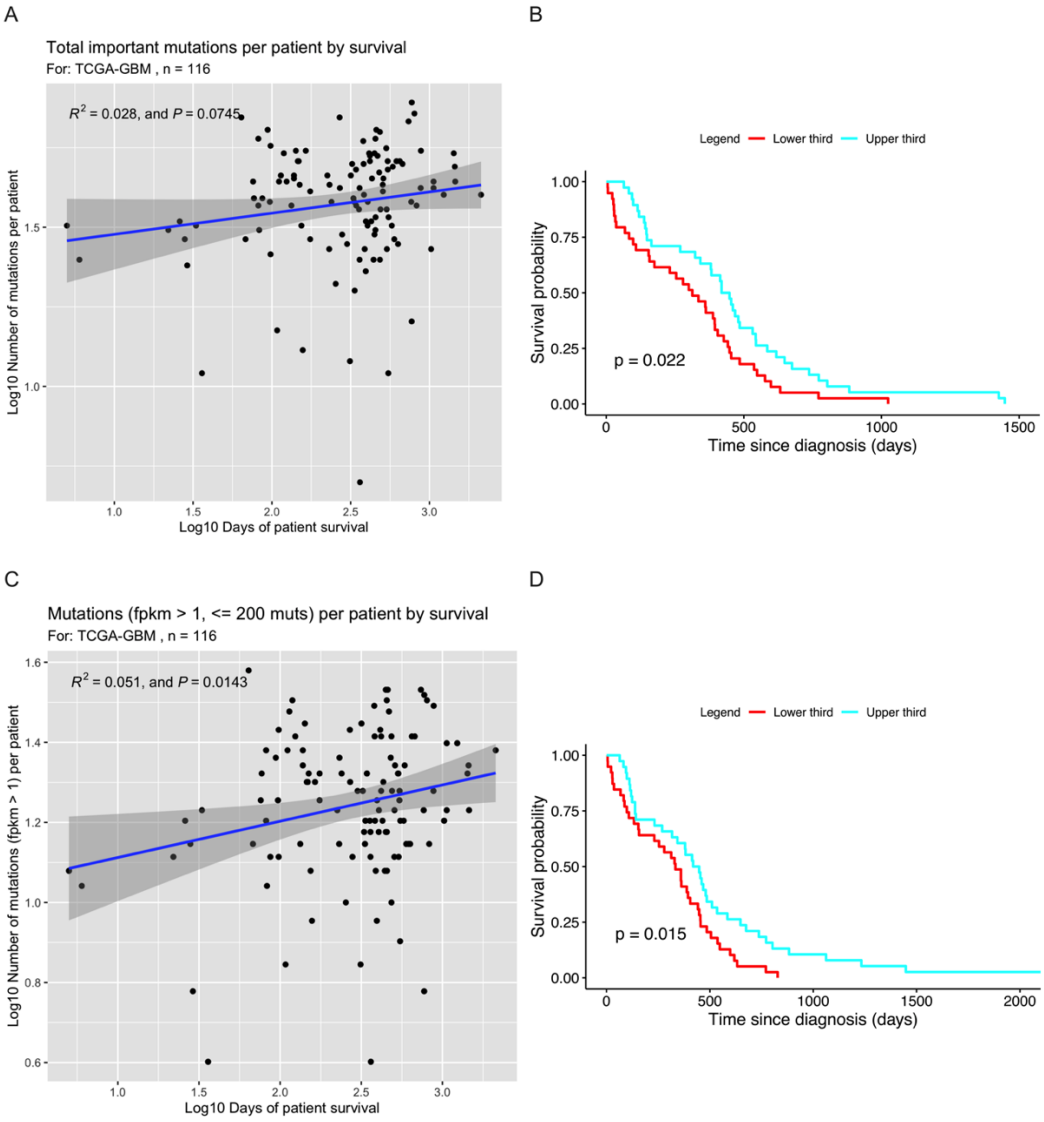


Figure 3.1 Number of expressed mutations is positively correlated with survival for patients included in TCGA. Scatter plots comparing (A) total non-silent mutation load and (C) expressed non-silent mutation load against overall survival from diagnosis. Kaplan-Meier plots comparing survival of patients in the upper third and lower third as separated by (B) total number of non-silent mutations and (D) number of expressed non-silent mutations.

If we then look at the missense mutations which are not expressed (FKPM<1), there are 45.7% that are methylated at their promoter sites (methylation score >0.1). In addition, we find that these not expressed mutations make up 16.3% of genes methylated in GBM gene set (Mueller *et al.*, 2007) and 8.8% of genes upregulated by DAC gene set (Kim *et al.*, 2006). This provides initial support to our hypothesis that there are a cohort of potential

neoantigen encoding mutations that are not expressed due to DNA methylation in GBM, which can be potentially upregulated following treatment with DAC.

3.2.2. Neoantigen prediction in U87MG and primary patient derived glioblastoma cell lines

Since we were interested in the mutations that were not/lowly expressed, we did not take in account expression data to perform this neoantigen prediction. Mutation data was obtained for U87MG from the Cancer Cell Line Encyclopaedia (CCLE, Broad Institute) and used to predict neoantigens. The top 9 potential neoantigen encoding mutations were selected and can be seen in table 3.1. The mutations were then confirmed through sanger sequencing (appendix).

Gene	Chromosome	Position	Mutation	Binding affinity (nM)	Reference	Binding affinity (nM)
MYRF	11	61541446	T	93.6	C	254.4
DNAH9	17	11837216	A	68.5	G	1119.5
EPHA3	3	89448536	T	5.1	G	18.8
OTOF	2	26684695	A	1.8	G	2.9
SLC6A12	12	313816	A	71.5	G	77.3
MASP1	3	186944297	G	3.3	T	5.3
OR2D2	11	6913503	C	30.2	T	96
CARD14	17	78164655	T	68.1	C	68.6
PTPN5	11	18754846	T	36.7	C	673

Table 3.1 Top 9 potential neoantigen mutations identified from U87MG. Mutation data was obtained from CCLE and neoantigens predicted from this. The top neoantigens as determined by HLA-A2 restricted priority score were selected and the mutations giving rise to these neoantigens are shown. Binding affinity refers to binding affinity of top predicted neoantigen and respective wild-type peptide.

HLA typing of patient samples can be found in the appendix. Four HLA-A02 patients (HGG2,7,11,13), for whom a primary cell line was established were chosen for WES and

neoantigen prediction. For Patients HGG7,11,13 WES of the primary tumour sample was also performed. The number of missense mutations and frameshift mutations can be seen in table 3.2. A comparison between primary cell line and tumour sample can be seen in figure 3.2.

Patient	**Missense**		**Frameshift**		**TMB**	
	Tumour	Cell line	Tumour	Cell line	Tumour	Cell line
HGG2	-	95	-	9	-	1.73
HGG7	34	39	11	6	0.75	0.75
HGG11	43	48	19	8	1.03	0.93
HGG13	49	49	27	7	1.27	0.93

Table 3.2 Tumour and cell line mutation numbers are similar. Table showing number of missense and frameshift mutations called for tumour samples and corresponding cell lines from WES. Tumour samples were sequenced at 150X depth and cell lines at 50X depth. No primary tumour sample was sequenced for HGG2 due to inadequate sample size. TMB – tumour mutational burden.

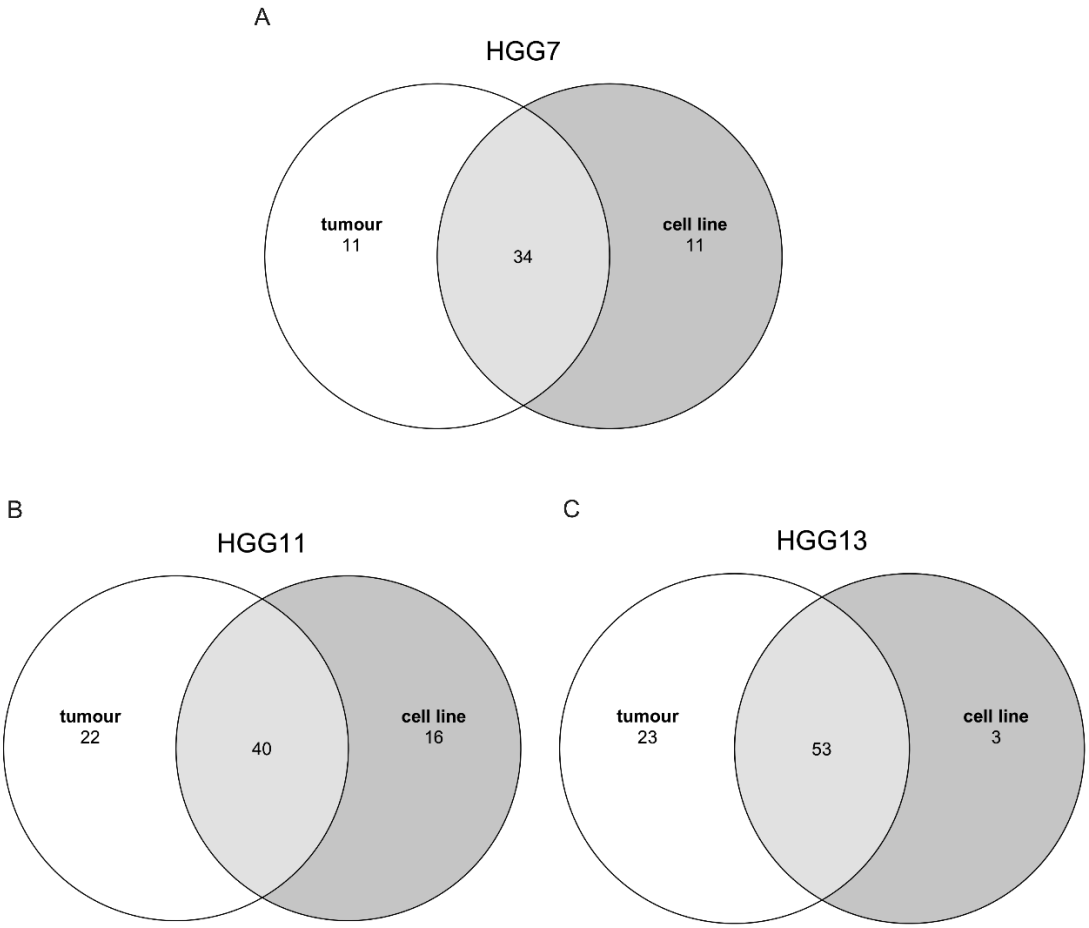


Figure 3.2 The majority of mutations sequenced are found in both primary tumour and derived cell line. Venn diagrams showing overlap of called mutations for both primary tumour samples and corresponding cell lines. (A) HGG7, (B) HGG11, (C) HGG13. Tumour samples were sequenced at 150X depth and cell lines at 50X depth. No primary tumour sample was sequenced for HGG2 due to inadequate sample size.

Neoantigens were then predicted and filtered by priority score $\geq$ 50 and a mean allele frequency (MAF) $\geq$5% for the mutated allele and a MAF $\leq$5% for the wild-type allele based on RNAseq analysis. This list was then further filtered on HLA-A02 binding for downstream functional experiments. In total 153 HLA-A2 binding peptides were selected arising from 104 mutations across the 4 patients (see appendix for full list).

3.2.3. Decitabine reduces growth but not viability

Treatment of U87MG tumour cells with 1µM of DAC leads to reduction in the rate of proliferation but does not significantly reduce the viability of tumour cells (figure 3.3). Therefore, any downstream analysis performed are on equally viable cells.

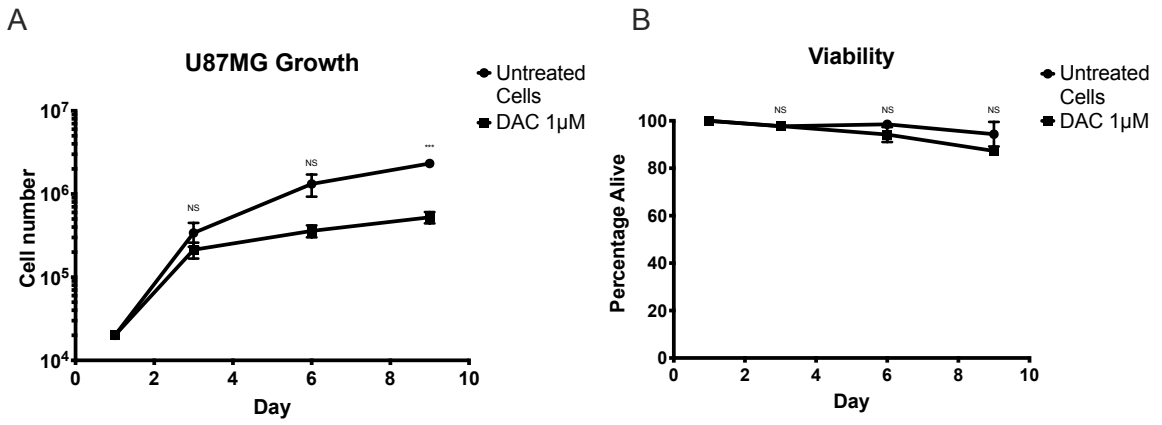


Figure 3.3 Decitabine treatment reduces cell proliferation but not cell viability. (A) Growth and (B) viability as measured by trypan blue staining. Significance as determined by 2-way ANOVA.

3.2.4. Tumour lines express MHC Class I and decitabine has minimal effect on expression levels

Decitabine is known to increase MHC class I expression (Barrero, 2017), whilst cancers are known to downregulate MHC class I as an immune escape mechanism (Marincola *et al.*, 1999; Sucker *et al.*, 2014). Therefore, to investigate both the baseline expression of MHC class I and any effect DAC treatment has on this, we perform flow cytometry analysis on the primary tumour cells. As can be seen in figure 3.4, there is high level expression of MHC Class I in all of our tumour lines also in untreated cells, which remains unchanged following treatment with DAC.

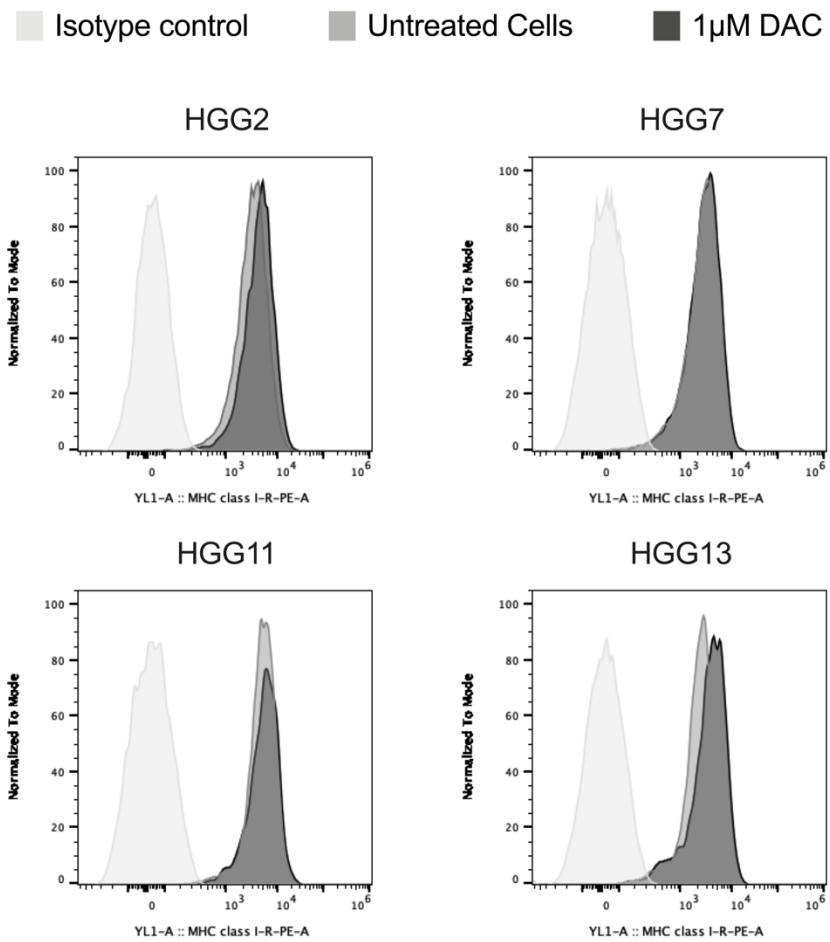


Figure 3.4 MHC Class I expression is high and unchanged following DAC treatment. Representative histograms, obtained by flow cytometry using a pan-class I antibody (clone W6/32) on HGG2,7,11,13 cell lines show high level of baseline expression, which is unchanged following treatment with DAC.

3.2.5. Decitabine increases potential neoantigen encoding mutations and cancer testis antigen expression in U87MG but not in normal human astrocytes

Analysis shows no or low baseline expression of 6/9 potential neoantigen encoding genes in the U87MG cell line. Following treatment with 1μM DAC, the expression of these genes encoding the mutations are significantly increased (figure 3.5A). The same can be seen for the CTA NY-ESO, SSX2, and PRAME in U87MG but not in normal human astrocytes (NHA) (figure 3.6B-D).

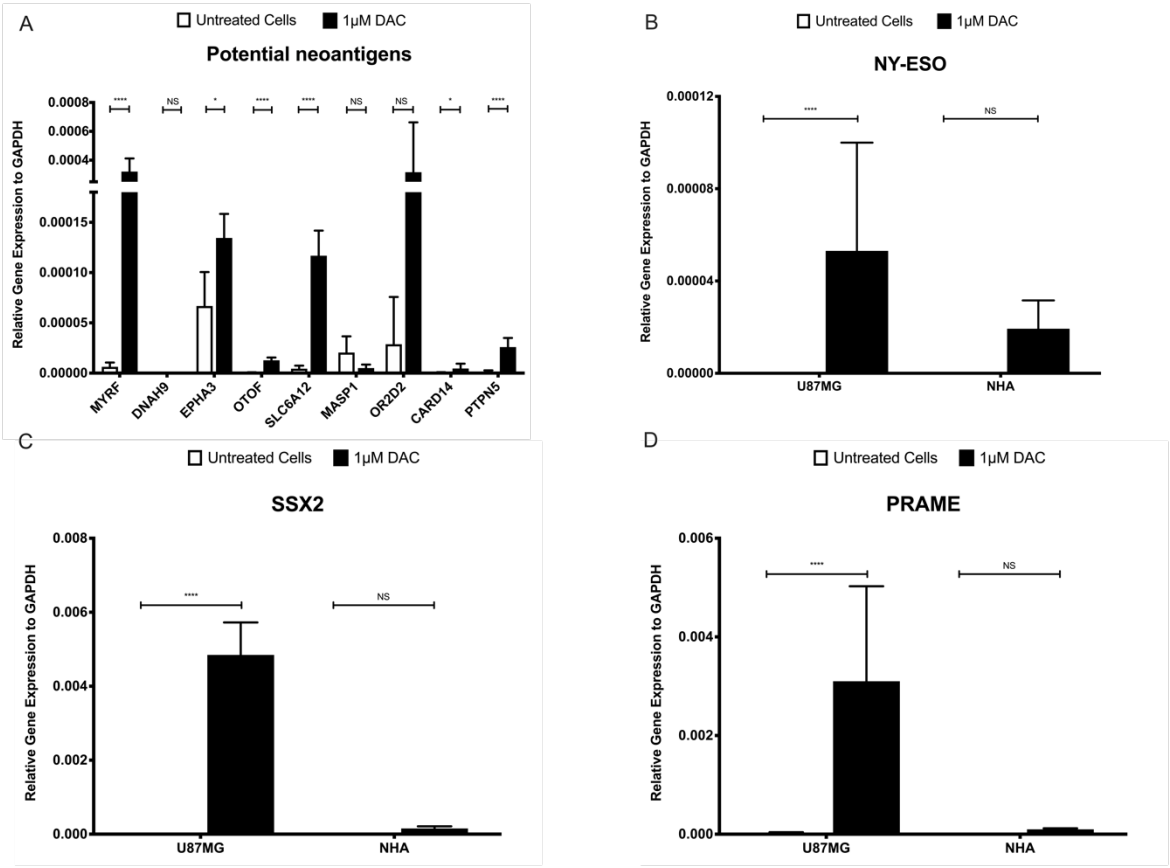


Figure 3.5 DAC treatment increases potential neoantigen and CTA expression in U87MG cell line but not NHA. Bar plots showing RTqPCR based expression of genes harbouring potential neoantigen encoding mutations (A) and CTA (B-D). Expression is shown as a relative expression to GAPDH. Data shown from 3 biological replicates each in technical triplicates. Significance as calculated by 2-way ANOVA.

In addition, targeted next generation sequencing performed on the mRNA transcripts of selected genes containing mutations shows following treatment with DAC, there is an increase in the mutated allele in comparison to the wild type for MYRF (figure 3.6).

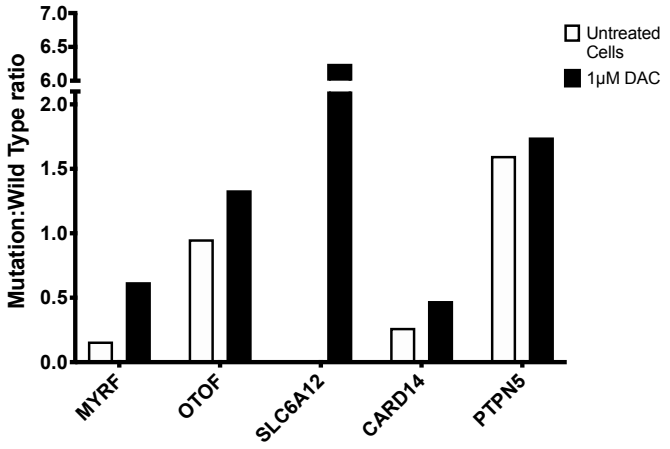


Figure 3.6 Mutated vs wild type allele ratio of expression of potential neoantigen-encoding genes for 1μM DAC treated cells is increased as compared to untreated control. Bar plots showing targeted RNAseq by NGS of cell lines +/- treatment with DAC. Ratio calculated by comparing number of reads of mutated allele compared to wild type reads. There were no reads detected for SLC6A12 in the untreated cell sample.

Western blot shows that the increase in transcription is also translated at the protein level. Figure 3.7 shows that there is no NY-ESO protein in untreated cells, but following 1μM DAC treatment, becomes unsilenced and is detectable. The same is true to the SLC6A12 protein, which is upregulated approximately 2.5x following treatment with DAC in the presence of a proteasome inhibitor.

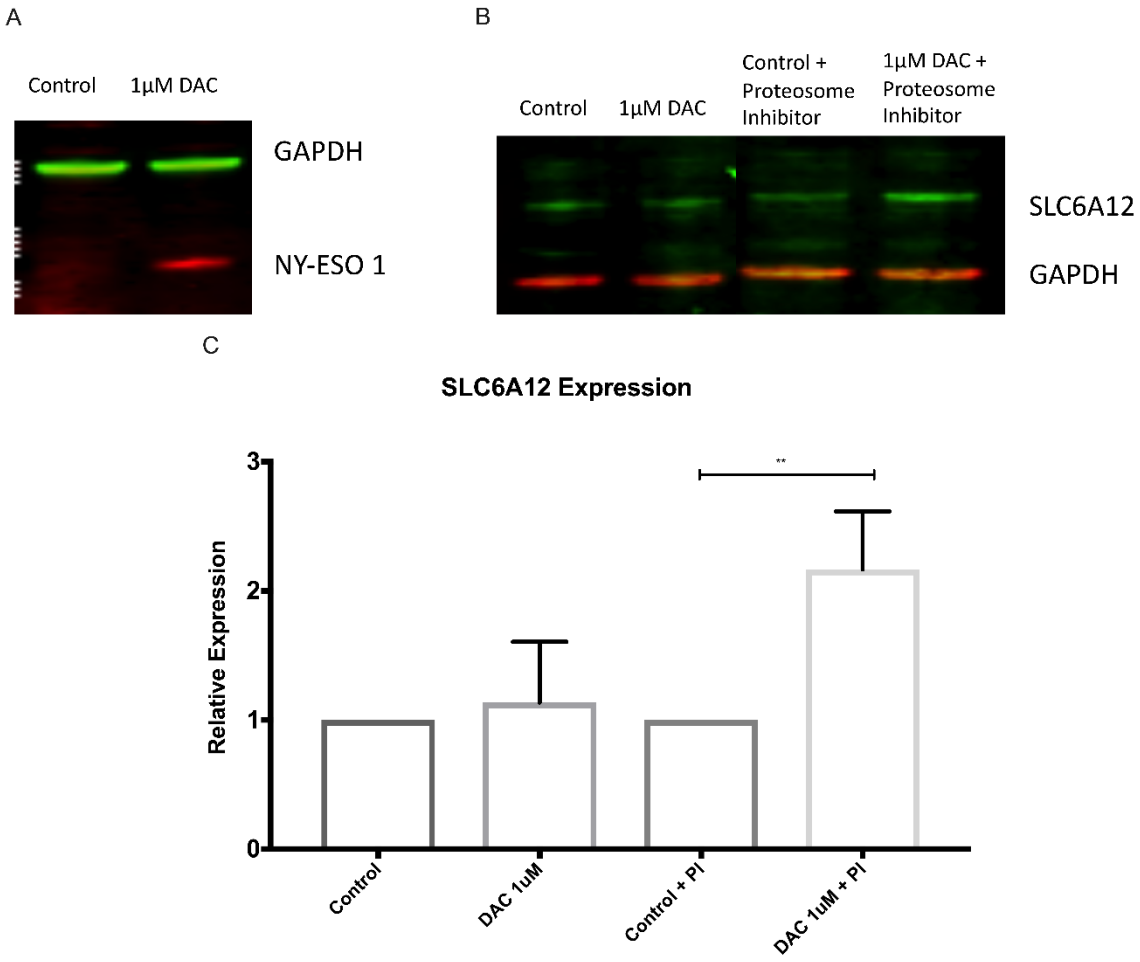


Figure 3.7 DAC treatment increases protein expression of both CTA and neoantigens. Representative Western blots showing expression of NY-ESO (A) and potential neoantigen harbouring protein, SLC6A12 (B). (C) Bar plot showing quantification of fluorescence seen in (B). Significance as determined by 2-way ANOVA.

3.2.6. Decitabine increases both neoantigen and cancer testis antigen transcription in primary patient cell lines

In order to investigate the effects of DAC on the whole transcriptional profile of glioblastoma cells, primary cell lines from HGG2,7,11 and 13 were treated with and without 1μM DAC and whole transcriptome sequencing was performed in triplicate. Principle component analysis (PCA) performed shows that the samples initially clustered based upon cell line and then by treatment (figure 3.8).

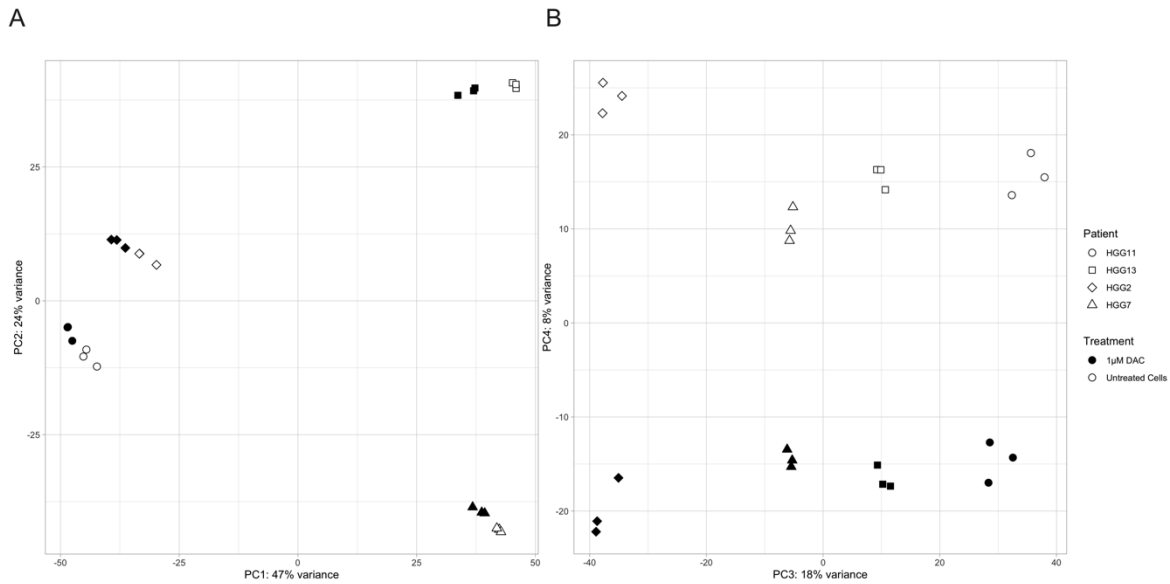


Figure 3.8 PCA analysis shows clustering of samples by cell line and treatment. PCA plots of RNAseq samples shows (A) the samples initially cluster based upon patient cell line and (B) secondly by treatment modality.

On gene set enrichment analysis (GSEA), some of the most enriched gene sets include genes methylated in GBM, and genes upregulated following treatment with DAC (figure 3.9). This is good control evidence to show that the DAC treatment was successful.

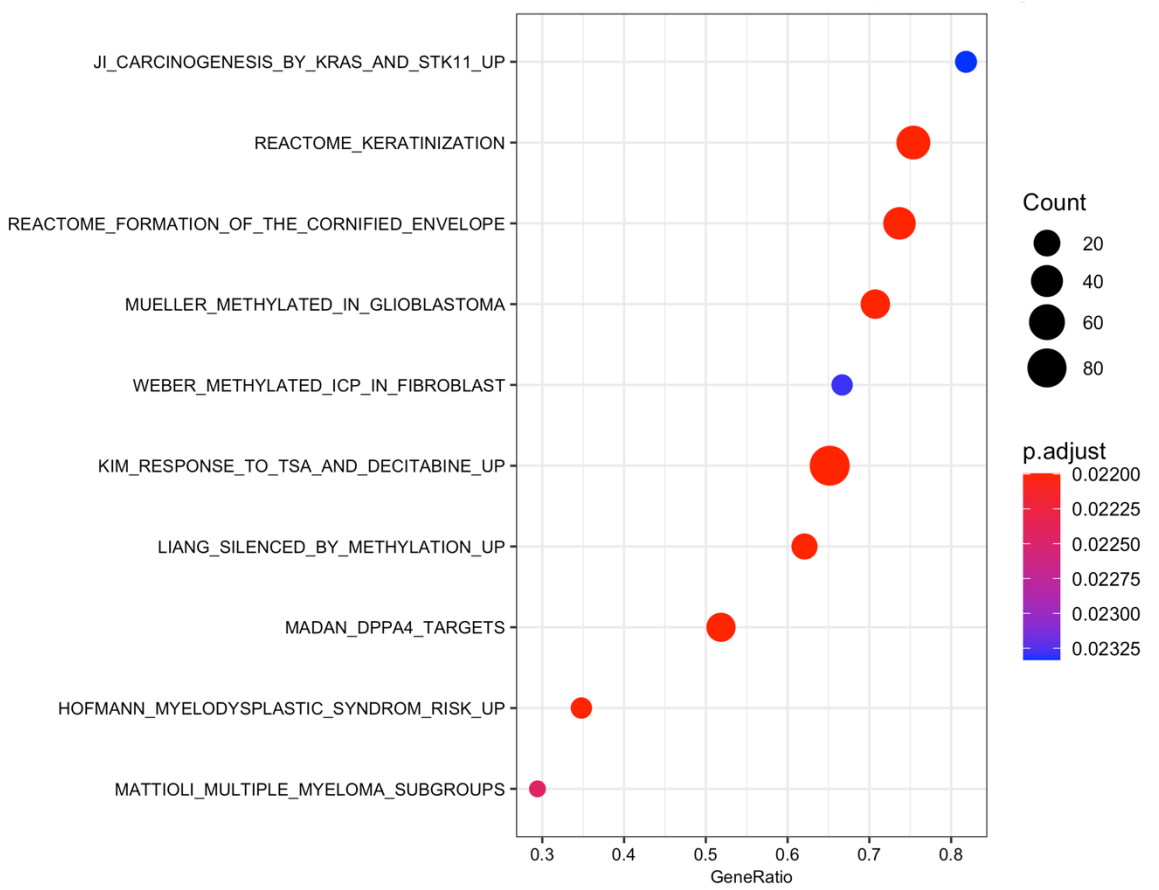


Figure 3.9 GSEA analysis of RNAseq results show enrichment in gene sets including genes methylated in GBM and genes upregulated by DAC. Dot plot showing most enriched gene sets following DAC treatment from MSigDB collection C2: curated gene sets.

We performed clustering analysis against transcriptomic subgroups previously described in GBM (Verhaak *et al.*, 2010) and show that we have 1 tumour from each subtype (HGG2 – mesenchymal, HGG7 – pro-neural, HGG11 – mesenchymal/neural, HGG13 – classical). Importantly, we find that DAC treatment does not change molecular subtype (figure 3.10).

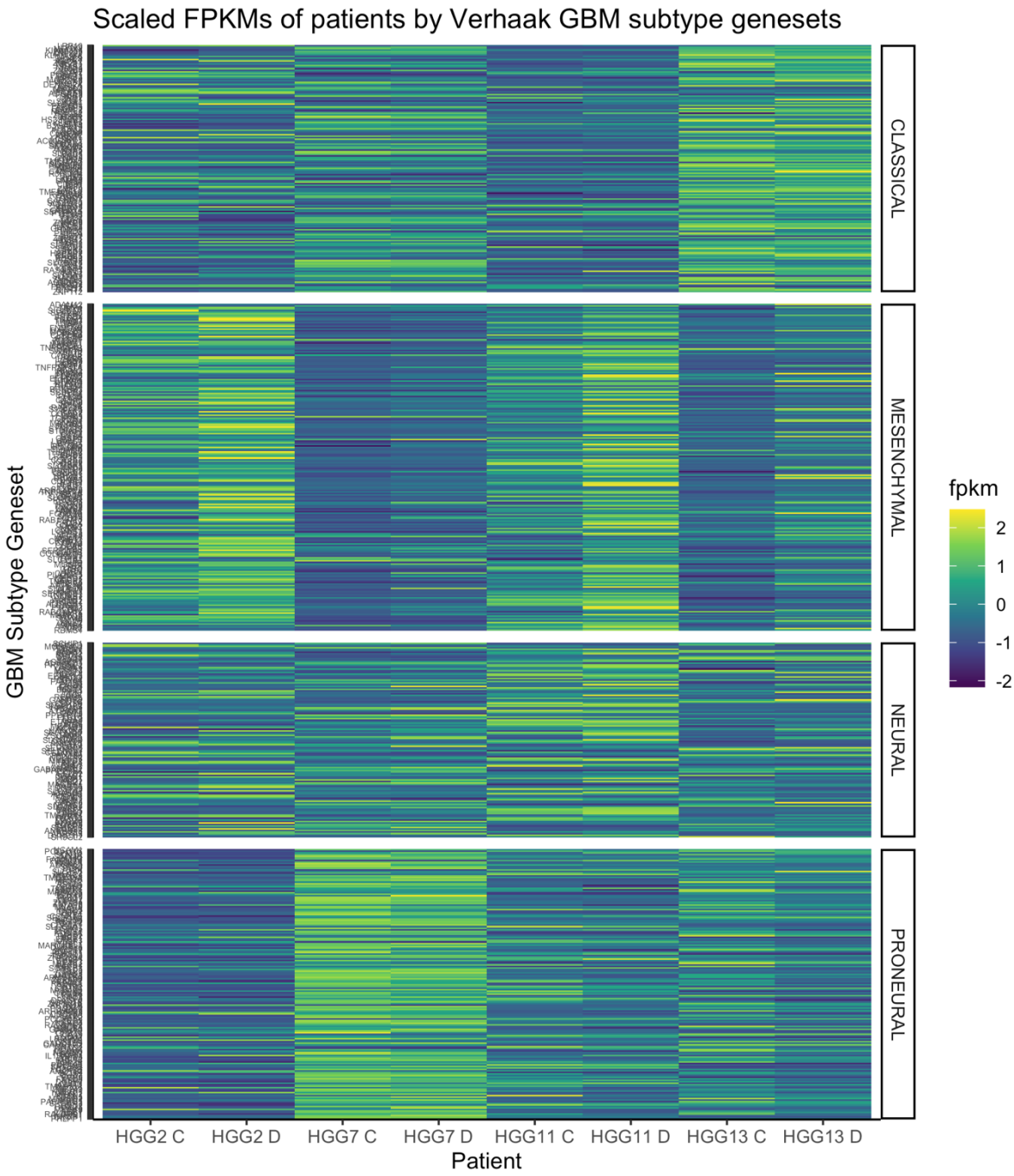


Figure 3.10 Classification of HGG tumour lines. Clustering of gene expression based on previously described molecular subtypes (Verhaak *et al.*, 2010). Y axis represents genes differentially expressed between different subgroups. HGG2 clusters to mesenchymal subtype, HGG7 clusters to proneural subtype, HGG11 clusters to neural and mesenchymal subtypes and HGG13 clusters to classical subtype.

Analysis of the expression of potential neoantigen encoding mutations in the cell lines that have not been treated with DAC shows that the majority of mutations are either not or lowly expressed (FKPM<1, figure 3.11).

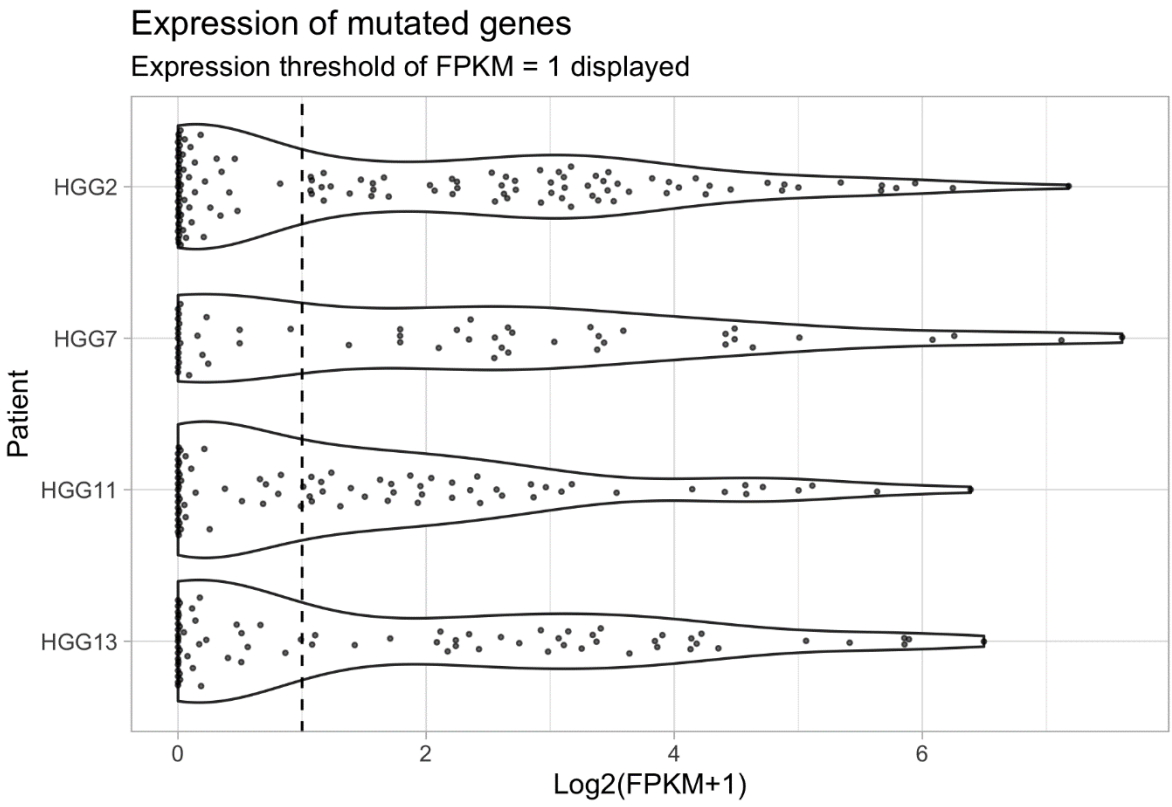


Figure 3.11 The majority of potential neoantigen encoding mutations are not/lowly expressed. Violin plot showing FKPM values of genes with potential neoantigen encoding mutations in primary cell lines not treated with DAC.

Comparing gene expression in cell lines treated with DAC to untreated cells shows that there are much more genes upregulated following DAC treatment than down regulated (figure 3.12A). Expression of potential neoantigen encoding genes shows that there is upregulation of 22/183 genes across the 4 cell lines. Although there is variability, there are potential neoantigens upregulated in all primary cell lines (figure 3.12B). Strikingly, DAC treatment had a major effect on the expression of CTA, with the majority of the most significantly upregulated genes being CTA (figure 3.12C).

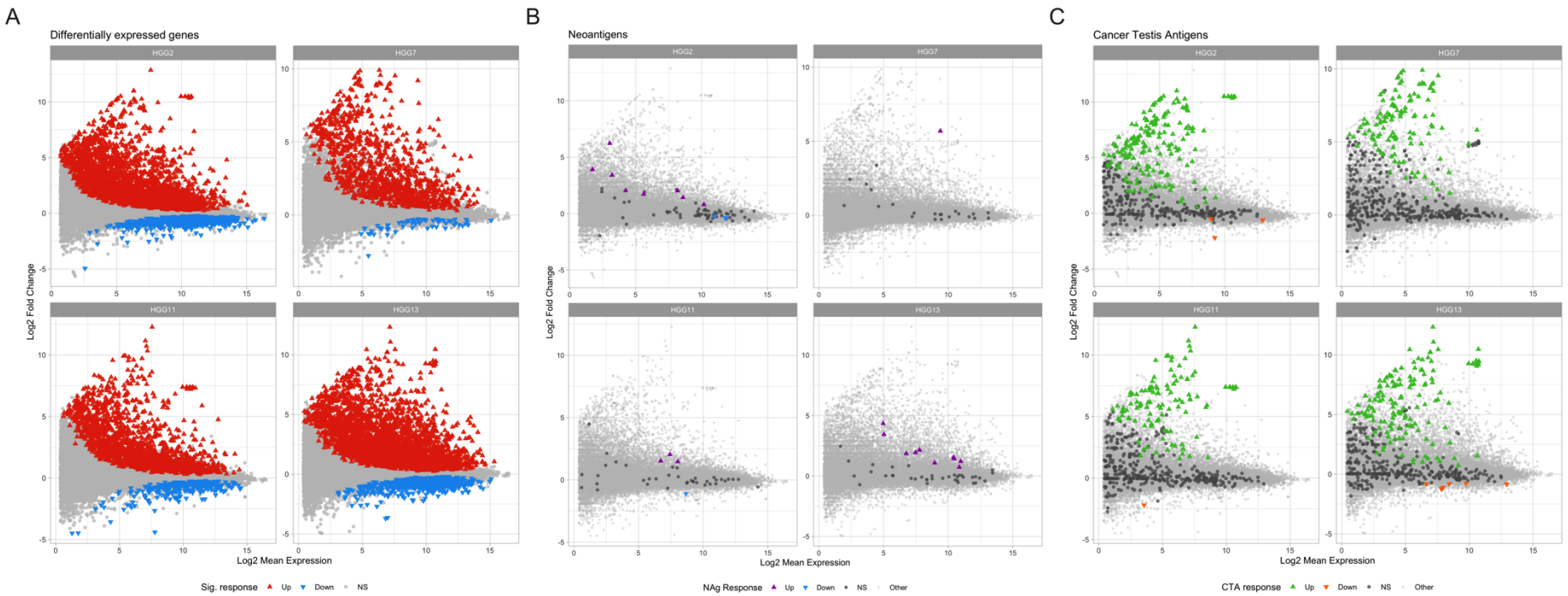


Figure 3.12 DAC treatment of primary GBM cell lines increases neoantigen and CTA expression. MA plots showing gene expression values and fold change of cells treated with 1µM DAC compared to untreated cells. (A) Overall there are much higher numbers of upregulated genes compared to downregulated genes. (B) There are potential neoantigens upregulated across all 4 cell lines. (C) There is an increase, across all 4 primary cell line, of a wide spectrum of CTA as determined by a composite of CT database (Herman and Baylin, 2003) and da Silva et al. (da Silva *et al.*, 2017). NAg – neoantigen, CTA – cancer testis antigen

3.2.7. Increased expression following decitabine treatment is due to DNA hypomethylation

Early passage primary GBM cell lines grown in culture are thought to reflect fairly accurately tumour biology *in vivo* (Pollard *et al.*, 2009). We wanted to investigate whether this was also true for the methylation status of our cell lines. In addition, although the mechanism of action of DAC is known, we wanted to ensure any increase in expression and presentation of neoantigens was due to DNA hypomethylation and not any off-target effects of the drug. To address this, we performed methylation analysis using EPIC 850k methylation array (Illumina) on 3 patients cell lines (HGG2, 11 and 13) and 2 patient *ex vivo* tumour samples (HGG11 and 13). Comparison of *ex vivo* tumour samples against untreated cell lines shows that there are no significantly differentially methylated probes (DMP; figure 3.13A). Analysis of control and DAC treated cell lines shows, in line with the mechanism of action of DAC, there were 271,566 significantly DMP from 19,231 genes, all of which were hypomethylated except 1 probe which was hypermethylated (figure 3.13B). Of these significantly hypomethylated genes, there were 534/1245 CTA and 144/185 neoantigens across the 3 patients. Looking individually at patient samples it can be seen that methylation of CTA and sample specific neoantigens is very similar between the *ex vivo* tumour sample and the untreated cell line and significantly lower in the DAC treated cell lines (figure 3.13C,D)

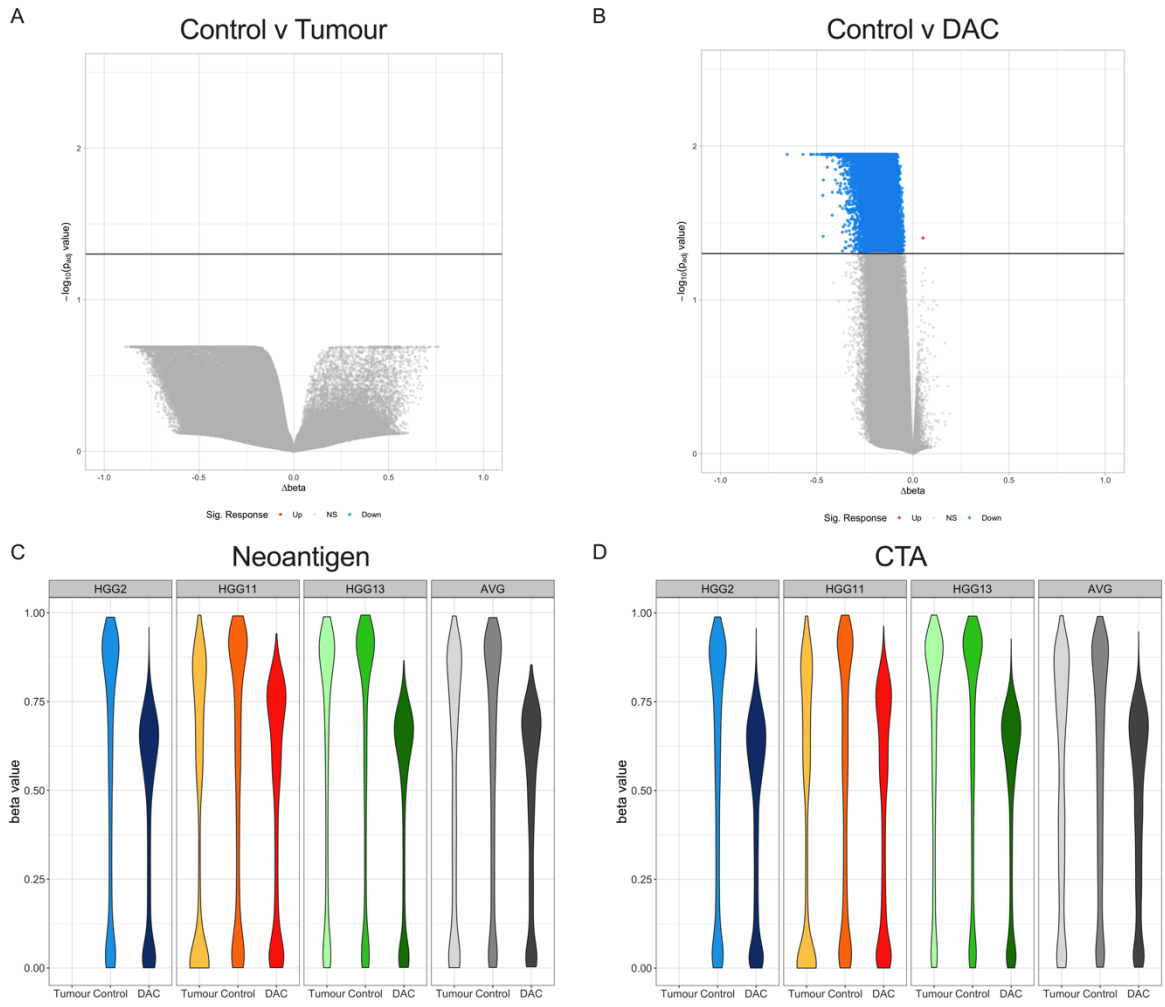


Figure 3.13 There is no significant difference in the methylation pattern of *ex vivo* tumour samples compared to the patient or primary cell line and DAC treatment of cell lines leads to global hypomethylation of the genome including significant decrease in methylation of CTA and neoantigens. (A+B) Volcano plot showing changes in methylation of individual probes between (A) control (primary cell lines) v tumour samples (*ex vivo* samples) and (B) control (primary cell lines) v DAC treated cell lines. (C+D) violin plots showing overall methylation of (C) genes harbouring potential neoantigen encoding mutations and (D) CTA in individual samples and averaged across the cohort.

3.2.8. Decitabine increases neoantigen and cancer testis antigen peptide presentation in primary patient cell lines

To understand whether the increased expression of CTA and neoantigen leads to an increase presentation of CTA and neoantigen epitopes on MHC Class I, potentially increasing the immunogenicity of the cell lines, we performed mass spectrometry analysis on peptides eluted from MHC class I for HGG13 to interrogate any changes in antigen

presentation following treatment with DAC. We found the length of peptides presented by MHC-I was similar in treated and untreated cells, with an expected enrichment in 9-mers (figure 3.14A). There was an enrichment of Leucine in position 2 and Leucine and Valine in position 9 (figure 3.14B). This is consistent with the finding that the majority of eluted peptides were bound to HLA-A02:01 (figure 3.14C). Following treatment with DAC there was an increase in the number of peptides presented on HLA-B08:01 (20.6% c.f. 16.3%).

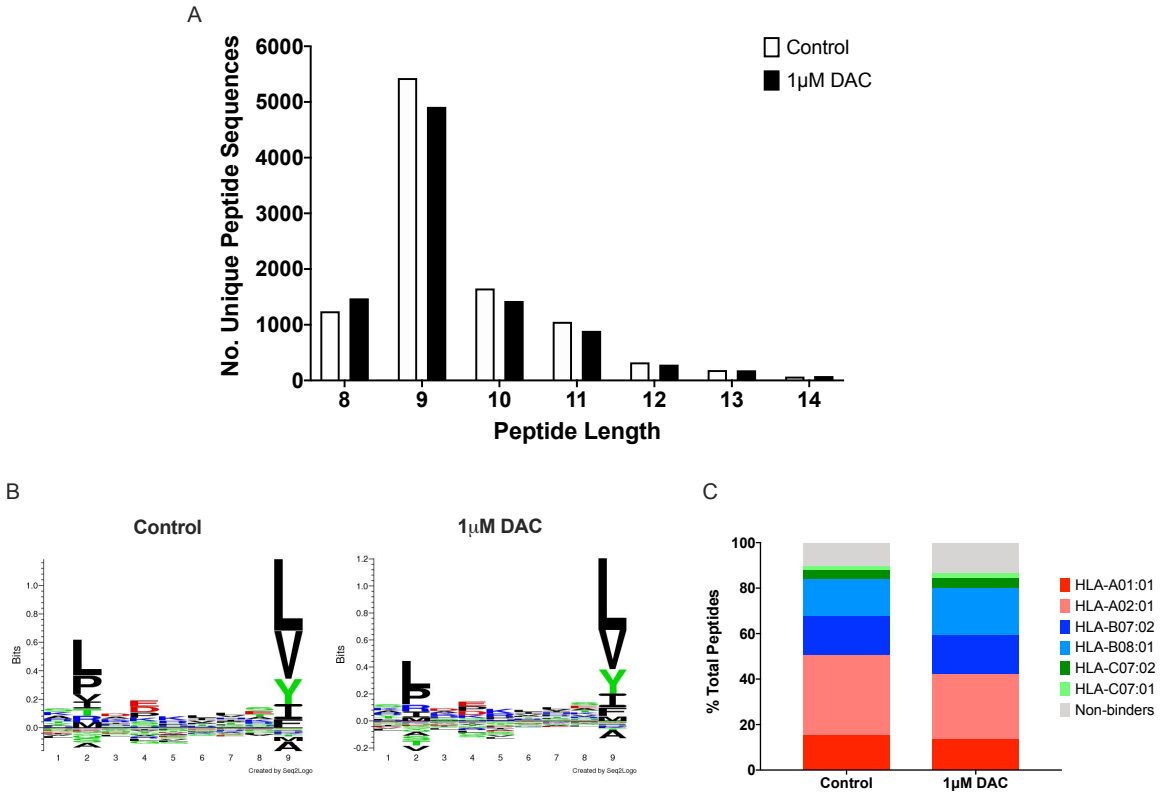


Figure 3.14 DAC treatment of HGG13 cell line increases presentation of HLA-B08:01 bound antigens. (A) Bar plot showing length of peptides identified by mass spectrometry on $1.7x10^9$ HGG13 cells +/- DAC treatment. (B) Plot showing broadly similar molecular structure of peptides. (C) Stacked bar chart showing proportion of peptides presented on the different MHC class I molecules.

3.3. Discussion

We initially explored the TCGA GBM dataset to validate our hypothesis and show that there is a trend towards increased survival with increased number of total non-silent mutations. Furthermore, if we take gene expression into account then there is a significant

relationship between total number of expressed mutations and overall survival. Importantly, 45.7% of mutations that are silent (FKPM<1) are also methylated (methylation score >0.1), showing that there are mutations whose expression we can potentially increase through hypomethylation.

Experimentally, we show that DAC treatment does significantly reduce tumour cell proliferation by day 9, although our experiments all utilise cells treated for 5 or 7 days for transcriptomic and proteomic/functional analysis respectively. Importantly, although there is a reduction in cell growth, there is no significant difference in viability of the treated cells and so any downstream experiments are performed using equally viable cells, which will be important in analysing T cell specific cell lysis.

We also quantified MHC class I expression on the tumour cell lines and show MHC class I is expressed in the tumour lines at baseline and there is no significant change in expression following DAC treatment. This is an important finding as tumours are known to downregulate MHC class I as an immune escape mechanism (Marincola *et al.*, 1999; Sucker *et al.*, 2014). Therefore, it is important to know that there is expression of MHC class I as without it, T cells cannot be activated regardless of expression of the antigens. It is also important to show that DAC does not significantly alter MHC expression because this may potentially act as a confounding factor in any functional experiments. In the absence of this we can be more confident that any increased activation of T cells following DAC treatment is due to increased expression of the antigen and not increased MHC class I.

In our cohort of 4 patients, we show that the tumour mutational burden (0.75-1.73 mutations per mega base pair) is similar to that reported in the literature. We show that the cell lines we derived have similar mutational profiles to the original tumour samples

obtained from the patient. In accordance with the literature, we were unable to predict many 'good' neoantigens per tumour sample, coming up with a list of 153 HLA-A2 binding peptides across all 4 patients.

RTqPCR for the 9 selected genes harbouring potential neoantigen encoding mutations in U87MG reveals that 6/9 genes are either not or very lowly expressed in untreated cells at baseline and that following treatment with DAC, 6/9 are able to be significantly upregulated (figure 3.5). Whilst RTqPCR is excellent and quantifying gene expression, it does not provide any information regarding whether it is the wild-type or mutated allele that is expressed and any increase in gene expression could just be due to changes exclusively in the wild-type allele. To address this, we performed targeted next generation sequencing to look at the relative allele frequencies seen in the transcriptome. We show that there is no decrease in the allele frequency of the mutated allele in any of the genes explored and there may be an increase in the mutated allele frequency for MYRF and only the mutated allele of SLC6A12 is transcribed. In addition, we also show upregulation of NY-ESO, SSX2 and PRAME as exemplar CTA. Importantly, we also show that, whilst DAC treatment does lead to un-silencing of CTA in normal human astrocytes, the levels of transcripts are ~10-fold less than that in tumour cells and this increase in transcripts may not be significant. To confirm this is the case, we also test this functionally as discussed in chapter 5.

Whole transcriptome sequencing of 4 primary cell lines shows that DAC, as expected, predominantly increases gene expression. Gene set enrichment analysis confirms that the upregulated genes are enriched for genes normally methylated in GBM and for genes that are known to be upregulated following DAC treatment. This acts as a good control showing

that DAC treatment, in our hands, yields similar results seen by other groups. The bias towards upregulation of genes makes analysis of change in gene expression difficult as a there is an increase in total transcripts in DAC treated cells, but the total sequenced reads is the same. Therefore, a similar FKPM may actually represent an increased expression level in the treated cells. This is something that we found difficult to control for and therefore our analysis of upregulated genes may be under representative of the true number. To overcome this uncertainty, we performed RTqPCR for selected mutation harbouring genes for which we were able to generate neoantigen specific T cells (chapters 4&5). Despite this limitation, we were able to show that there were potential neoantigen encoding mutations that are upregulated in all patient samples, especially HGG2 and 13. The effect on CTA is particularly striking, with CTA making up the majority of the most significantly upregulated genes.

We performed methylome analysis and show that overall methylation of the primary cell lines is similar to the *ex vivo* tumour sample for the 2 patients studied, providing good evidence to support the veracity of our *in vitro* model. We go on to show that DAC treatment leads to global hypomethylation of the genome including 534/1245 CTA and 144/183 neoantigens across the 3 patients studied. This provides evidence to support our hypothesis that the increase in immunogenic antigen expression following DAC treatment is due to hypomethylation.

Finally, we also show that this increase in transcription is also translated at the protein level. In U87MG we are able to detect NY-ESO by western blot following treatment with DAC whereas it is silent beforehand and there is a 2.5-fold increase in protein levels of SLC6A12 following DAC treatment. Immunopeptidomics analysis of HGG13 shows that

there is a subtle change in peptides presented on the cells, with a 4% increase in peptides presented by HLA-B08:01.

In this chapter we have shown that there are potential neoantigen encoding mutations as well as CTA that are not/lowly expressed in GBM and we are able to increase the expression of these immunogenic antigens through DAC treatment. In the next chapter, I describe our efforts to obtain neoantigen and cancer specific T cells to perform functional experiments to address whether increased expression of these immunogenic antigens is sufficient to increase immunogenicity of these tumours.

4. Isolating peptide and tumour specific T cells

4.1. Introduction

In the previous chapter I showed that the expression of both neoantigens and CTA can be increased by treating cell lines with DAC. In order to further test our hypothesis that the increase in the expression immunogenic antigens leads to increased immunogenicity it is imperative to be able to isolate neoantigen and CTA specific T cells.

Identifying and isolating tumour specific, and more recently neoantigen specific, T cells has been a focus of many groups including ours. The success rates of this is variable and relies on many factors. Currently, the most commonly used neoantigen prediction algorithms, including our own, are based upon NetMHCPan (Jurtz *et al.*, 2017), which estimates the binding affinity of specific peptide sequences to the MHC class I molecules. Much of these calculations are done based upon binding affinities of known peptide sequences, however, the latest version also takes into account data from eluted ligands (Jurtz *et al.*, 2017). These advances have led to significant improvements in our ability to predict if a peptide will bind to an MHC class I molecule. Whilst being a predominating factor in neoantigen prediction, binding affinity to MHC is only a small part of the antigen presentation pathway and there are many other factors that are important but less well characterised and for which we are much less accurate at modelling. Cytosolic proteins are processed and cleaved in the proteasome in a non-random fashion (Nielsen *et al.*, 2005; Gomez-Perosanz, Ras-Carmona and Reche, 2020). These peptides are then actively transported into the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP 1/2) (Townsend and Trowsdale, 1993). From there, peptides are loaded on to MHC class I molecules in the ER through interactions with tapasin (Kelly and Trowsdale, 2019). Once translocated onto the

cell membrane, the peptide-loaded MHC class I then has to interact with the TCR present on CD8 T cells with sufficient affinity to elicit downstream TCR signalling and activation. Therefore, current prediction algorithms still have plenty of room for improvement, with success rates variable between 1-50%, depending on number of potential neoantigens tested (A. M. Bjerregaard *et al.*, 2017). More accurate predictions will confer multiple advantages, namely, 1) given a set number of potential neoantigens, more accurate predictions will allow researchers to focus their testing on fewer potential neoantigens, reducing both time and monetary costs; 2) if there are a large number of potential neoantigens, for example in melanoma, it can increase the number of positive hits for the same number of potential neoantigens tested and finally, 3) when used in a therapeutic vaccination setting, increased prediction accuracy will increase the likelihood of eliciting a relevant neo-antigen immune response.

In this chapter I present the different techniques we utilised to obtain peptide and neoantigen specific T cells both from the patients and also HLA-A2+ healthy donors. I also present work looking to integrate more aspects of the antigen presentation and recognition pathway into our neoantigen prediction algorithms.

4.2. Results

4.2.1. Isolating peptide specific T cells from patient PBMC

To identify and isolate neoantigen specific T cells, we predicted neoantigen peptides using our in-house prediction pipeline (TUNAPASTA). In brief, patient mutations, HLA-types and TPM expression data are input and processed to determine a ranked list of neoantigens based upon the published MuPeXI pipeline (A.-M. Bjerregaard *et al.*, 2017). We stimulated

autologous patient PBMC with the relevant predicted neoantigen peptide pools for all 4 patient samples (HGG2 – 51 peptides in 2 pools, HGG7,11,13 – 34 peptides each in single pools). Through this method, we were able to generate 1 peptide specific T cell clone, which stained positively for the relevant peptide loaded tetramer (figure 4.1).

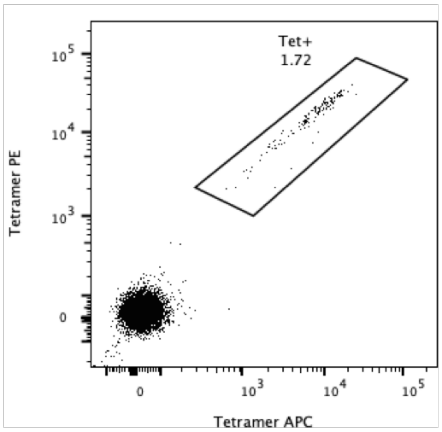


Figure 4.1 Tetramer positive cells were sorted by flow cytometry. Representative plot of tetramer-APC/PE+ cells that were sorted in bulk and expanded. Cells were pre-gated on CD3+/CD8+ population.

4.2.2. In vitro priming of healthy donor PBMC is capable of generating a wide range of peptide specific T cells

In order to augment the numbers of peptide specific T cells, we moved to an *in vitro* priming method for healthy HLA-A2+ donor PBMC. In brief, we stimulated CD14- fraction of PBMC with peptide pulsed autologous moDCs in the presence of IL12 and IL7 for 10-14 days followed by a second round of stimulation with peptide pulsed moDCs in the presence of IL-2. Through this method, we were able to identify and isolate 34 unique T cell clones that were reactive to 36/160 different peptides (figure 4.2).

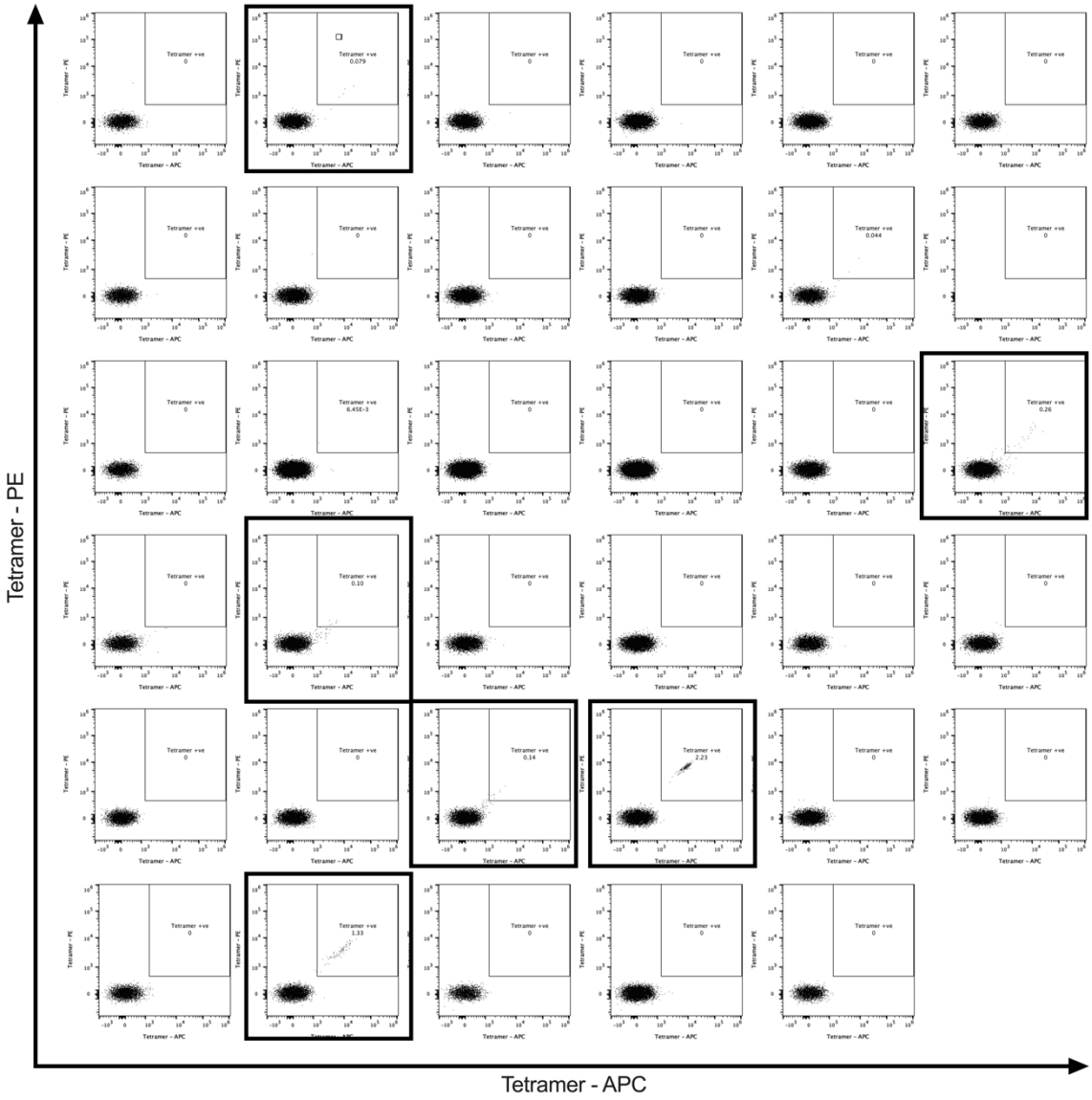


Figure 4.2 *In vitro* peptide priming of healthy donors to generate to peptide specific T cells. Representative dot plots from tetramer screening of *in vitro* primed CD8 T cells. Plots represents screening from 1 healthy donor stimulated against predicted neoantigen peptides for HGG13. Cells are stained against individual peptide loaded tetramers conjugated to APC and PE. Tetramer+ gate represents APC/PE double positive cells. Tetramer+ cells that are then sorted are highlighted. Population shown are pre-gated on CD3+/CD8+ve cells.

Using the results of the screening, tetramer+ T cells were isolated and expanded for subsequent usage. These T cell clones showed reactivity to the relevant peptide in a concentration dependent manner as measured by intracellular cytokine staining (TNFα) and degranulation (CD107a) (figure 4.3).

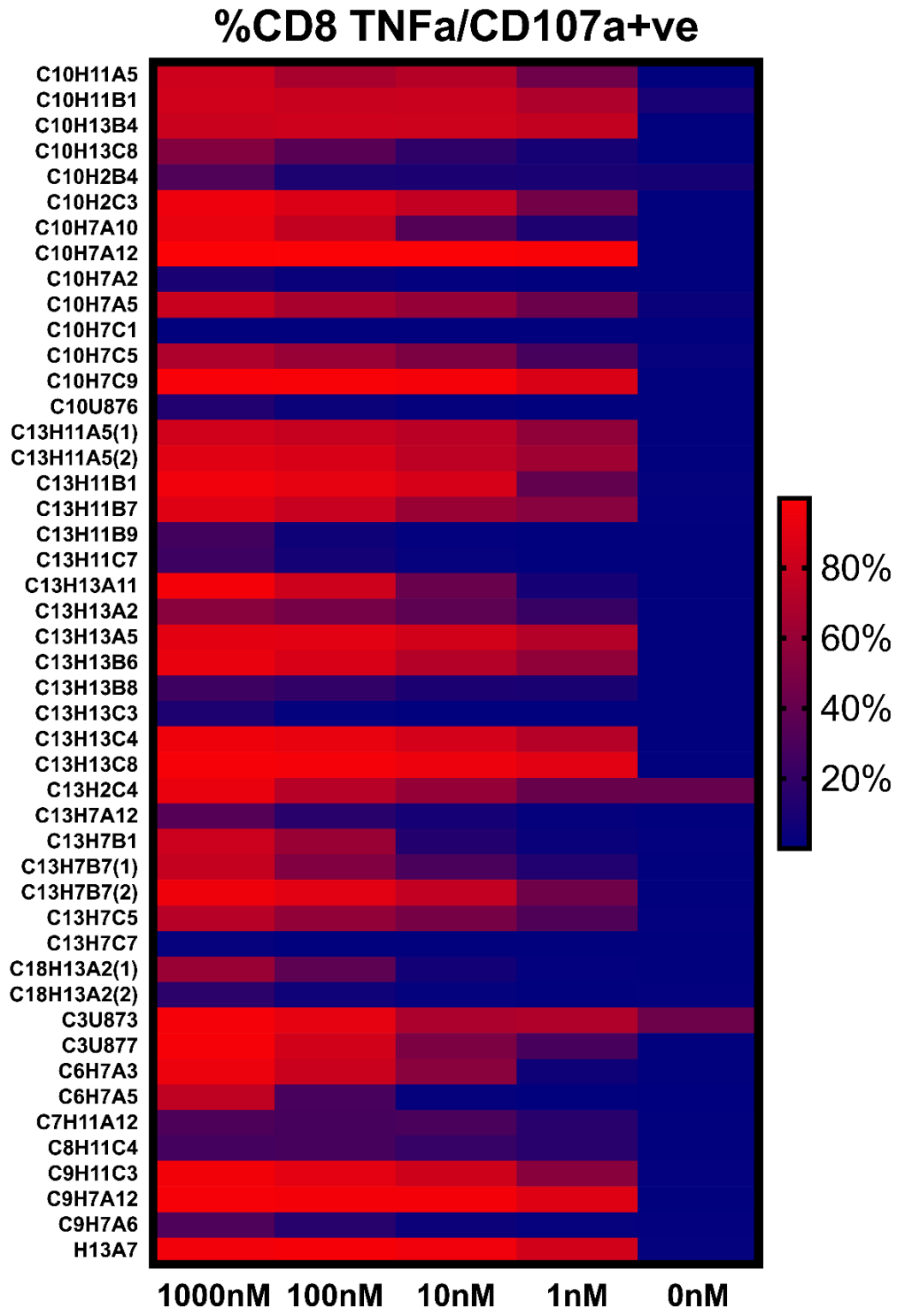


Figure 4.3 Peptide specific T cells isolated show reactivity against the peptide in a concentration dependent manner. Heat map showing response to the relevant peptide, at concentrations ranging from 0-1000nM, for all T cell clones isolated. Response is measured as % cells TNFα+/CD107a+ and depicted in different shades of grey, with darker grey meaning higher responses. Cells are pre-gated on CD3+/CD8+ve. Data shown is from 3 biological replicates each performed as technical duplicates.

4.2.3. Addition of an immunogenicity prediction model increases the accuracy of immunogenic peptide prediction

To investigate whether our results are consistent with computational tools used to predict the immunogenicity of peptides, we then computed our results using an immunogenicity prediction (iPred) algorithm (Pogorelyy *et al.*, 2018). The iPred algorithm is trained on the physicochemical properties (average of the Kidera feature scores) of all the amino acid residues in a given antigen. Figure 4.4 (A, B) shows that the peptides for which we were able to generate specific T cells (immunogenic) had a significantly higher immunogenicity score. The iPred scoring system showed good accuracy with an area under the curve (AUC) score of 0.725 (figure 4.4C). In addition, whilst there was good overlap for some of the peptides with our TUNAPASTA priority score, there was a significant second population of immunogenic peptides that had a priority score <70 but an iPred score >0.75 (figure 4.4D), suggesting that the addition of the iPred immunogenicity score to the priority score further enhances our prediction accuracy.

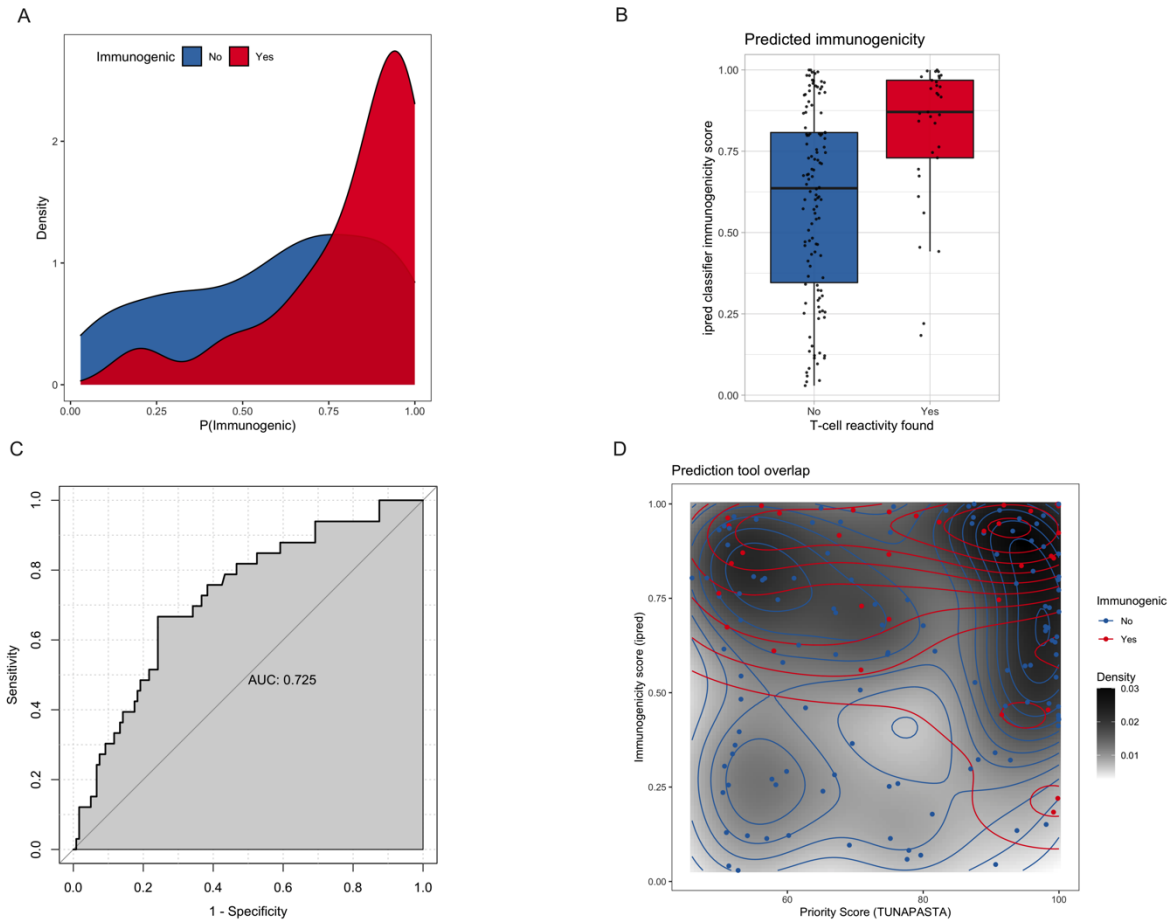


Figure 4.4 Immunogenicity score increases accuracy of immunogenic antigen prediction. (A) histogram and (B) box and whisker plots showing immunogenicity score for peptides in which there was no T cell response (red) compared to peptides eliciting a positive T cell response (blue). Peptides that elicited a positive T cell response have a significantly higher immunogenicity score (P=0.0000191). (C) Receiver operating characteristic (ROC) curve showing accuracy of the iPred prediction algorithm on our dataset. (D) Density plot comparing TUNAPASTA priority score to iPred immunogenicity score.

4.2.4. Identifying neoantigen specific T cells from peptide specific T cells

As these peptides were predicted *in silico* to be neoantigens, we then further tested the T cells against the relevant cell lines to test if the predicted neoantigens were presented by the cell lines. From the 35 T cell clones, we were able to confirm that 6 of them were reactive, indicating that those were true neoantigen specific T cells and able to recognise the tumour (table 4.1).

T cell clone	Peptide	Cell line	Gene	Mutation
C13H2C4	YITAAYVE**V**	HGG2	MCM7	chr7:100094259 T>C
C18H2D4	**I**LFQCMPVPV	HGG2	WDR91	chr7:135208809 C>T
C8H11C4	KLPDLW**C**KI	HGG11	LAMA3	chr18:23909188 C>T
C18H13A2	I**M**EEVWYFL	HGG13	DNAH3	chr16:20963718 G>A
H13A7	F**L**GITNML	HGG13	FAM122a	chr9:68780820 A>T
U87 6	KELPM**F**TYRV	U87MG	MYRF	chr11:61541446 C>T

Table 4.1 T cell clones and their respective neoantigen. T cell clones isolated and the neoantigen peptide they are reactive against. Table also highlights the cell line where the mutation is present. Bold amino acid represents the mutated residue.

We then tested whether these T cells were neoantigen specific or whether they had any cross reactivity with the wild-type peptide. It can be seen in figure 4.5 that there was 1 T cell clone that was cross reactive to the wild-type peptide but the remaining 5 were neoantigen specific.

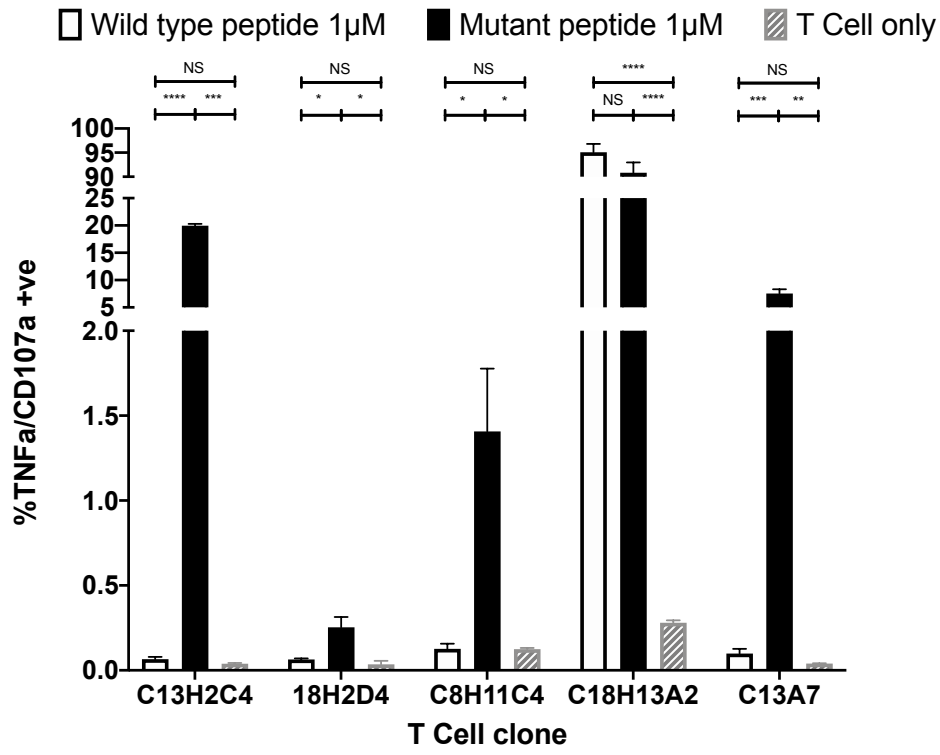


Figure 4.5 Most neoantigen specific T cells isolated do not recognise the wild-type peptide. Bar plot showing response of neoantigen specific T cells when co cultured with U87MG cells pulsed with 1µM of either mutant or wild-type peptide. Response is measured as % cells TNFα+/CD107a+. Cells pre-gated on CD3+/CD8+. 4/5 neoantigen specific T cells are show specificity for the mutated peptide with 1 clone (C18H13A2), showing cross reactivity for both mutant and wild-type peptides. Data shown is of technical triplicates representative of 2 biological replicates. Significance as determined by multiple student t-tests.

4.2.5. Reporter Jurkat cells transduced with TCR retain similar properties as primary T cells

There is a chance that T cell clone activation may be due to a non-TCR:MHC mediated mechanism such as through the NKG2D (Prajapati *et al.*, 2018; Kim and Shin, 2019). In order to assess the specificity of the TCR, we sequenced the T cell clones and transduced a selection of the TCRs into reporter J8 Jurkat lines. These reporter cells have enhanced-GFP expression driven by a NFAT-binding promoter and we co-stained these cells with CD69 for increased specificity.

To test and optimise the reporter system, we initially transduced the 1G4 TCR that is responsive to a NY-ESO derived epitope that has previously been described in great detail by our laboratory (Chen *et al.*, 2005). We tested the J8 reporter cells and primary T cells in parallel against both the naturally occurring SLLMWITQC (9C) peptide as well as the high affinity SLLMWITQV (9V) peptide. We find that although the absolute numbers vary, the trends and patterns seen are similar in both types of cells (figure 4.6).

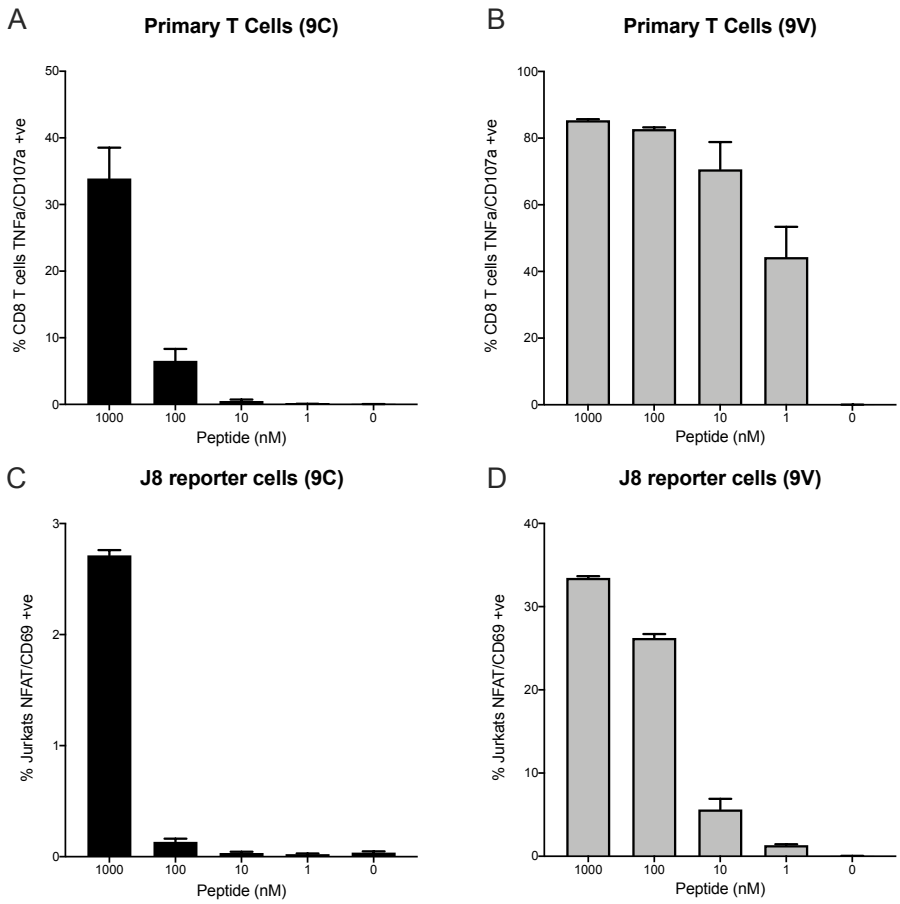


Figure 4.6 Reporter J8 cell lines show similar activation profiles to primary T cells. Comparison between activation of primary 1G4 T cells and reporter J8 cell line transduced with 1G4 TCR shows similar activation profile between the cells lines to both naturally occurring 9C peptide and high affinity 9V peptide. Activation as measured by CD107a+/TNFα+ cells in primary cells and NFAT-GFP+/CD69+ cells in J8 reporter. Cells pre-gated on CD8+ population for primary T cells and mouse TCRβ+ population for J8. Data shown is from 3 technical triplicates representative of 2 biological replicates.

4.2.6. Identifying tumour specific T cells through mixed tumour lymphocyte culture

To further increase the pool of tumour specific T cells, we utilised a MLTC method for an additional patient, HGG19. PBMCs were stimulated twice using irradiated autologous tumour cells +/- DAC and then single cell sorted using the IFNγ and TNFα capture assays (Miltenyi; figure 4.7). Sorted T cells were either subjected to scTCRseq and TCR transduction for further downstream functional testing. In total, 7 unique TCRs were identified and testing revealed that 2 of these were tumour reactive.

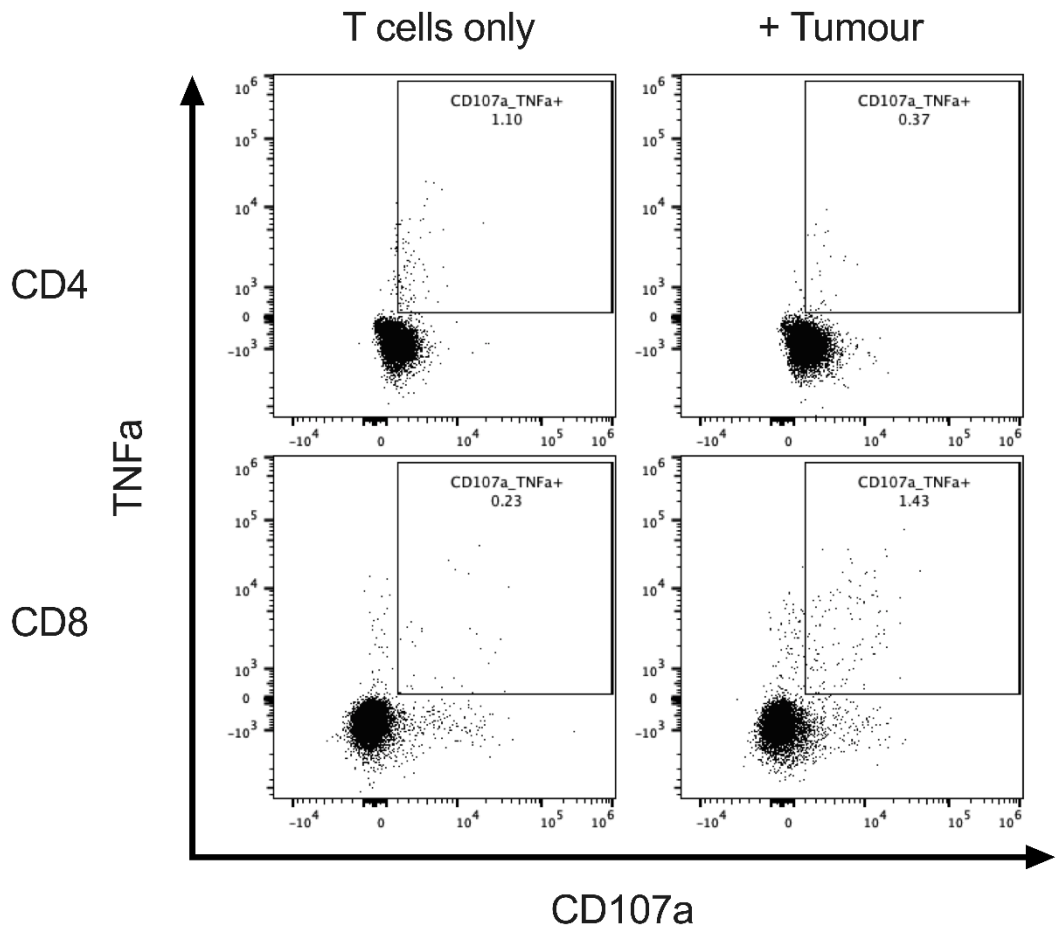


Figure 4.7 MLTC generates tumour reactive T cells. Representative dot plots of flow cytometry analysis of PBMC following a minimum of 2 rounds of stimulation co-cultured against autologous tumour line or PBMC alone. Activation as determined by CD107a+/TNFα+ cells. Single cell sorting was performed on the CD107a+/TNFα+ cohort as labelled. All cells pre-gated on CD3+/CD8+ population. Representative plots of 2 biological replicates.

4.3. Discussion

Identification of neoantigen specific T cells through use of peptides is limited by the accuracy of neoantigen prediction, which varies between 0.92% and 50% (figure 4.8). Therefore, in order to maximise potential yield, the more peptides tested the better, but there is a cost limitation to this. We were further limited to HLA-A2 binding peptides only due to the availability of specific MHC tetramers in order to isolate the cells following stimulation. Our overall success rate of finding peptide specific T cells was 22.5% (36/160), which compares favourably with the literature. However, when we look at success for

neoantigen specific T cells, the figure was 3.75% (6/160) which, although low, is not dissimilar to the other studies testing similar numbers of predicted neoantigens (figure 4.8).

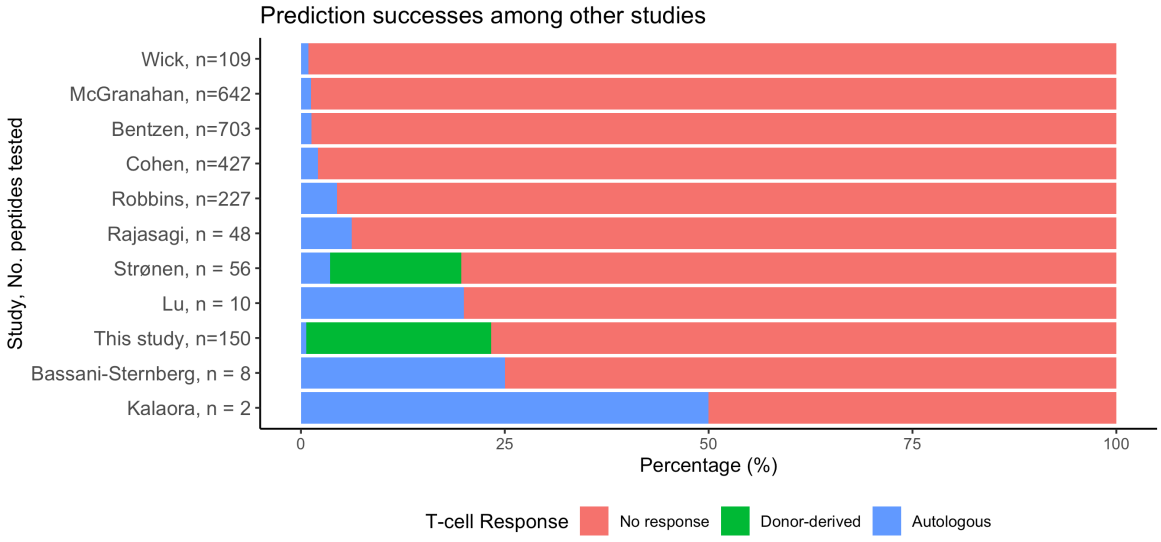


Figure 4.8 Neoantigen prediction accuracy varies significantly between studies. Neoantigen prediction accuracy as calculated by number of neoantigen specific T cells identified from predicted peptides. Accuracy varies between 0.92% and 50%. N = number of predicted neoantigens tested. Data adapted from Bjerregaard et al. (A. M. Bjerregaard *et al.*, 2017).

The most enriched sample to try and identify such cells would be from the TIL fraction. However, due to sample size and the relatively low number of TIL present in the sample we were not able to try this. We attempted to expand TIL *ex vivo* for 2 patients as per Zacharakis *et al.* (Zacharakis *et al.*, 2018) but were also unsuccessful with this. This is likely due to the low numbers of TIL and the fact they are all highly exhausted (Woroniecka *et al.*, 2018). Due to these limitations, we were reliant on the patients PBMC to try and identify neoantigen specific T cells, but this had a low rate success. This is unsurprising as we know that GBM is a non-immunogenic tumour so the likelihood of finding such T cells in the blood is very low. The one T cell clone we were able to isolate was neoantigen and tumour specific, but we were not able to significantly expand this T cell line compared to T

cell clones generated from healthy donors, suggesting again that these tumour specific T cells are likely exhausted *in vivo*.

We did have better success through *in vitro* priming of healthy donors' PBMC following a protocol adapted from Strønen et al. (Strønen *et al.*, 2016; Ali *et al.*, 2019), identifying 34 TCRs specific to 36 different peptides. Interestingly, if we apply an immunogenicity prediction algorithm (iPred) that takes into account the biochemical properties of the amino acids in the peptide sequence on top of the neoantigen priority score we were able to increase the prediction accuracy even further. This has implications in the future as it would be possible to generate the same number of peptide specific T cells from fewer peptides, reducing cost. For tumours, such as melanoma, where there are often thousands of predicted neoantigens and it is not feasible to purchase all of them, this additional step could help in selecting which peptides to test in order to maximise yield.

The priority score and immunogenicity prediction predict whether the peptide will bind the MHC and the likelihood of binding to a TCR respectively. However, there are many other factors upstream in the antigen presentation pathway that will also affect the likelihood that a predicted peptide is a true neoantigen. These include the expression of the mutation, proteasomal processing of protein to generate the peptides (Nielsen *et al.*, 2005; Gomez-Perosanz, Ras-Carmona and Reche, 2020), and the antigen presentation machinery (Townsend and Trowsdale, 1993; Kelly and Trowsdale, 2019), which all play a part in determining whether the peptide will be generated from the protein and whether it is processed and presented on the MHC molecule. All of these are not factored in with our calculations and explains the low conversion of peptide specific T cell to true neoantigen specific T cell (6/35).

Although we can get much greater success with the technique, a major downside to utilising the allogeneic derived T cells is that we are unable to show that these neoantigen specific T cells are present endogenously in the patients to be activated following DAC treatment in future chapters. There is a school of thought that claims that T cells, by their very nature, are cross reactive and that any 1 TCR is capable of recognising many peptides with varying affinity (Sewell, 2012). Therefore, it is likely that there will be an endogenous TCR that is capable to recognise any peptide as long as it is sufficiently 'foreign' from a self-antigen. As we have found T cells that respond specifically to these neoantigens, suggesting they are indeed sufficiently 'foreign', it may indeed be likely that similar T cells will exist within the patients themselves although we are unable to show this is the case.

To try and address this, we utilised a different approach to generate tumour specific T cells. By utilising a MLTC technique, we were able to generate 2 tumour specific T cells. The advantage of this technique is that we do not need to rely on any neoantigen predictions and are also not limited to peptides loaded a restricted number of HLA haplotypes. This technique also has the potential to generate T cells restricted to MHC-class II and non-classical antigen presenting molecules. Any T cells isolated will be reactive to an antigen that is capable of being processed and presented by the tumour cells. Utilising this method, we are able to isolate endogenous pre-existing tumour specific T cells and investigate whether they show increased reactivity to tumour cells treated with DAC. However, there are 2 downsides to this approach: 1) we are only able to detect T cells reactive to tumour antigens that are expressed and potentially may miss some neoantigen specific T cells to antigens that are silent/lowly expressed, the T cells we would be looking for; 2) once T cell clones are generated we are unable to easily identify their antigen so cannot conclusively say their increased activation is due to increased neoantigen or CTA expression. To address

the first issue, we treated some of the tumour cells with DAC before irradiation to try and maximise yield. The second issue is harder to overcome but as shown in the following chapter we test these T cells against other partially HLA matched cell lines to show the responses seen are tumour line specific.

In this chapter I have shown that we are able to generate neoantigen specific T cells through both peptide stimulation of autologous PBMC as well as *in vitro* priming of HLA matched healthy donor PBMC. We were also able to identify and isolate tumour specific T cells through a MLTC technique. In the next chapter, I describe our experiments utilising these T cells in functional assays against tumour cell lines to address our hypothesis that increased expression of neoantigens and CTA following DAC treatment is sufficient to induce increased T cell mediated activation and killing.

5. Increased expression of neoantigens and cancer testis antigens leads to increased tumour immunogenicity

5.1. Introduction

There is increasing recognition of the importance of both CTA and neoantigens as prognostic markers as well as biomarkers of response to immunotherapy. CTA expression in tumours has been shown to be a prognostic marker for various cancers including GBM (Scanlan *et al.*, 2002; Novellino *et al.*, 2004; Freitas *et al.*, 2013). Freitas *et al.* studied the expression of 30 selected CTA and found 4 to be expressed frequently (>15%) and that expression of >3-4 CTA led to a more favourable prognosis in GBM (Freitas *et al.*, 2013). In addition, much work has focused on the expression on neoantigens in cancers. It has been shown that the tumour mutational burden and by extension, neoantigen burden is directly linked to the efficacy of checkpoint blockade (Schumacher and Schreiber, 2015; Yarchoan *et al.*, 2017; Yarchoan, Hopkins and Jaffee, 2017; Keenan, Burke and Van Allen, 2019). More recent studies suggest that it is not just the number of neoantigens but also the clonality that plays an important role with more clonal neoantigens eliciting a better immunological response (Mcgranahan *et al.*, 2016) and that immunoediting plays a role in their expression (Rosenthal *et al.*, 2019). Therefore, by increasing the expression of such immunogenic antigens, then tumours should become more immunogenic and capable of eliciting an anti-tumour immune response. We have shown in chapter 3 that treatment of GBM cell lines with DAC leads to increase the expression of potential neoantigens and a plethora of many CTA . This should, in theory, make the tumours more immunogenic and the target of an increased adaptive immune response.

The anti-tumour response of the adaptive immune system was thought to be predominantly mediated by CD8 cytotoxic T lymphocytes (CTL) recognising tumour through peptides presented on MHC class I (Farhood, Najafi and Mortezaee, 2019). In addition, peptide loading on MHC class I has been much better characterised and prediction algorithms for presentation are more mature. Therefore, in the previous chapter, our efforts were predominantly focused on identification and isolation of these CTLs for testing against our tumour lines. As they become more studied, there is now greater recognition of the importance of CD4 T cells in the anti-tumour response both in helping prime an efficient CD8 CTL response as well as having direct cytolytic activity themselves (Tran *et al.*, 2014; Borst *et al.*, 2018; Zander *et al.*, 2019; Brightman *et al.*, 2020). Therefore, we also identified, isolated, and investigated CD4 T cells in our MLTC experiments.

In this chapter we utilise the tumour and neoantigen specific T cells obtained in chapter 4 as well as CTA specific T cells available in our group to investigate T cell activation and killing of our tumour lines before and after treatment with DAC. In addition, we transduced a selection of TCRs into J8 reporter cells to provide further evidence that T cell activity is dependent on the TCR:MHC interaction.

5.2. Results

5.2.1. Increased expression of potential neoantigen and cancer testis antigen in U87MG leads to increased T cell recognition and killing in a MHC dependent fashion

We obtained HLA-A2 restricted CTA specific CTL to NY-ESO and SSX2 from Dr Jili Chen and Dr Victoria Woodcock. In the absence of DAC, the cell lines do not express CTA (figure 3.5B) and do not induce degranulation and cytokine production by CTLs following co-culture (figure 5.1A). Following treatment, both NY-ESO and SSX2 are expressed and subsequently activate these CTLs. This effect is blocked with a pan MHC class I antibody (W6/32). In addition, the increase in activation translates into CTL mediated killing with increased killing following co-culture with DAC treated tumour cells, which again is reduced following the addition of W6/32 (figure 5.1B).

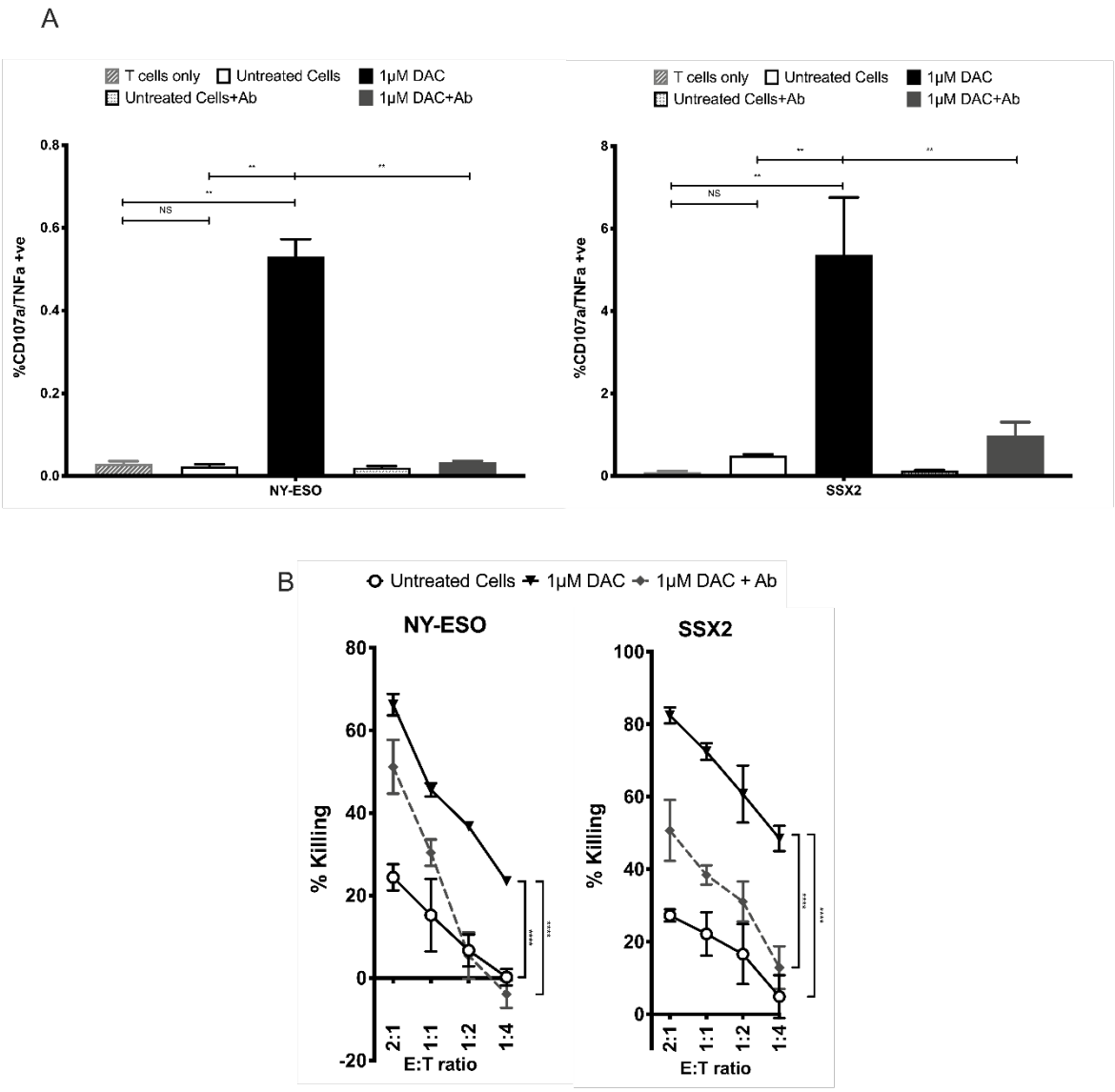


Figure 5.1 Increased expression of cancer testis antigens leads to increased CTL activation and killing in a TCR-MHC dependent fashion for U87MG cell line. (A) **Bar plots show** activation of NY-ESO and SSX2 specific CTL as measured by TNFα+/CD107a+ cells by flow cytometry. All cells pre-gated on CD3+/CD8+ population. (B) Plots show NY-ESO and SSX2 specific CTL mediated killing at decreasing effector to target ratio (E:T) as measured by LDH release against primary cell lines. Data shown is from technical triplicates representative of a minimum of 3 biological replicates. Statistical significance as calculated by 2-way ANOVA. Ab – W6/32 MHC class I blocking antibody.

We were also able to isolate a CTL specific to a peptide generated from the mutation in MYRF (U87 6). Using this CTL, we find increased CTL activation and killing following treatment with DAC (figure 5.2).

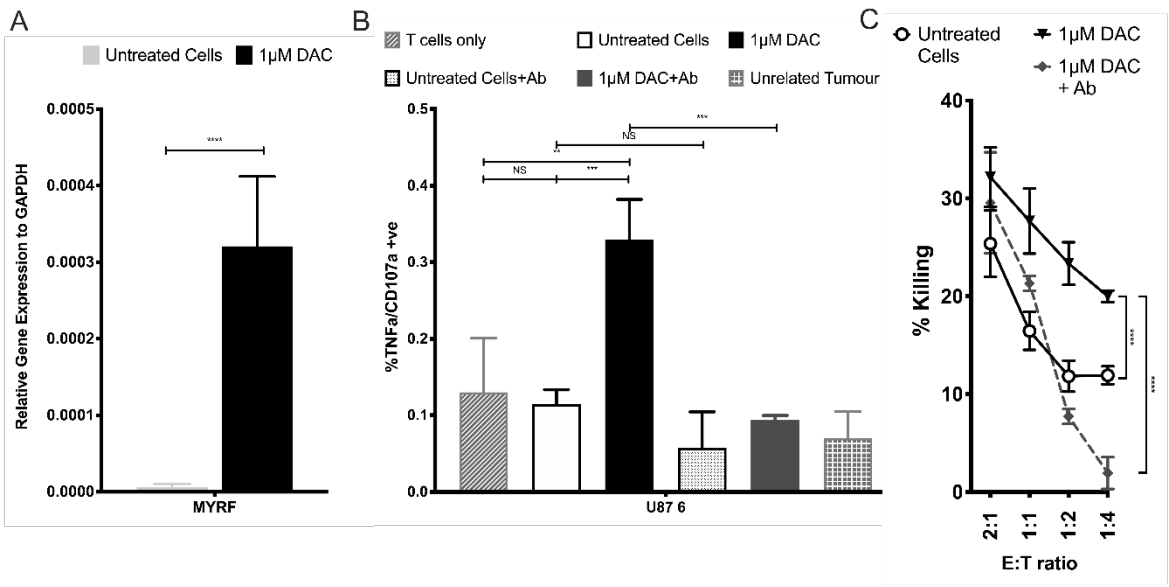


Figure 5.2 Increased expression of neoantigen leads to increased CTL activation and killing in a TCR-MHC dependent fashion for U87MG cell line. (A) Bar plot showing relative gene expression to GAPDH as measured by RTqPCR. Expression is represented as $2^{-\Delta\Delta CT}$ and statistical significance as calculated by paired two-tailed student t test. (B) CTL activation as measured by TNFα+/CD107a+ cells by flow cytometry. All cells pre-gated on CD3+/CD8+ population. Statistical significance as calculated by 2-way ANOVA. (C) CTL specific lysis as measured by LDH release at decreasing effector to target ratio (E:T). Data shown is from technical triplicates representative of a minimum of 3 biological replicates. Statistical significance as calculated by 2-way ANOVA. Ab – W6/32 MHC class I blocking antibody.

5.2.2. Increased neoantigen and cancer testis antigen expression leads to increased T cell activation and killing in an MHC dependent fashion in primary patient derived cell lines

Of the 5 neoantigen reactive CTLs, 4 were shown to be neoantigen specific (table 4.1), so, on those 4, we performed RTqPCR to quantify change in gene expression following treatment with DAC. It can be seen in figure 5.3 panels A, D, G, and J that 3/4 mutations (HGG2 – MCM7, WDR91; HGG11 – LAMA3) were significantly upregulated with the other mutation expression unchanged (HGG13 – FAM122a).

CTL activation is cell line specific, increased following DAC treatment if the gene is upregulated and is abrogated with MHC class I blocking antibody (figure 5.3 B, E, H, K). Increased CTL activation also leads to CTL mediated killing (figure 5.3 C, F, I). For CTL clone

H13A7, that recognises the neoantigen produced from HGG13 FAM122a mutation, there is no increase in CTL activation or killing following treatment with DAC, mirroring the unchanged mRNA level (figure 5.3 J,K,L).

We also quantify, by RTqPCR, change in expression of the CTA SSX2 and NY-ESO for which we have HLA-A2 restricted CTLs. We show variable, but increased expression across the cell lines (figure 5.4 A,D). In addition, we also obtained an HLA-A3 restricted PRAME specific CTL from Dr Jili Chen. None of the 4 patient cell lines or U87MG were HLA-A3+ but on testing of other primary cell lines, there were 3 that were HLA-A3+ (HGG19, 27 and 28). Therefore, we also performed RTqPCR on these cell lines for PRAME and show similar results (figure 5.4 G).

Again, there is increased CTL activation following co-culture with tumour cells treated with DAC (figure 5B, E, H) leading to increased CTL mediated killing, which is reduced in the presence of MHC class I blocking antibody (figure 5.4C, F, I). Of note, there is no significant change in expression of NY-ESO in HGG11 following DAC treatment and correspondingly, no CTL activation or killing.

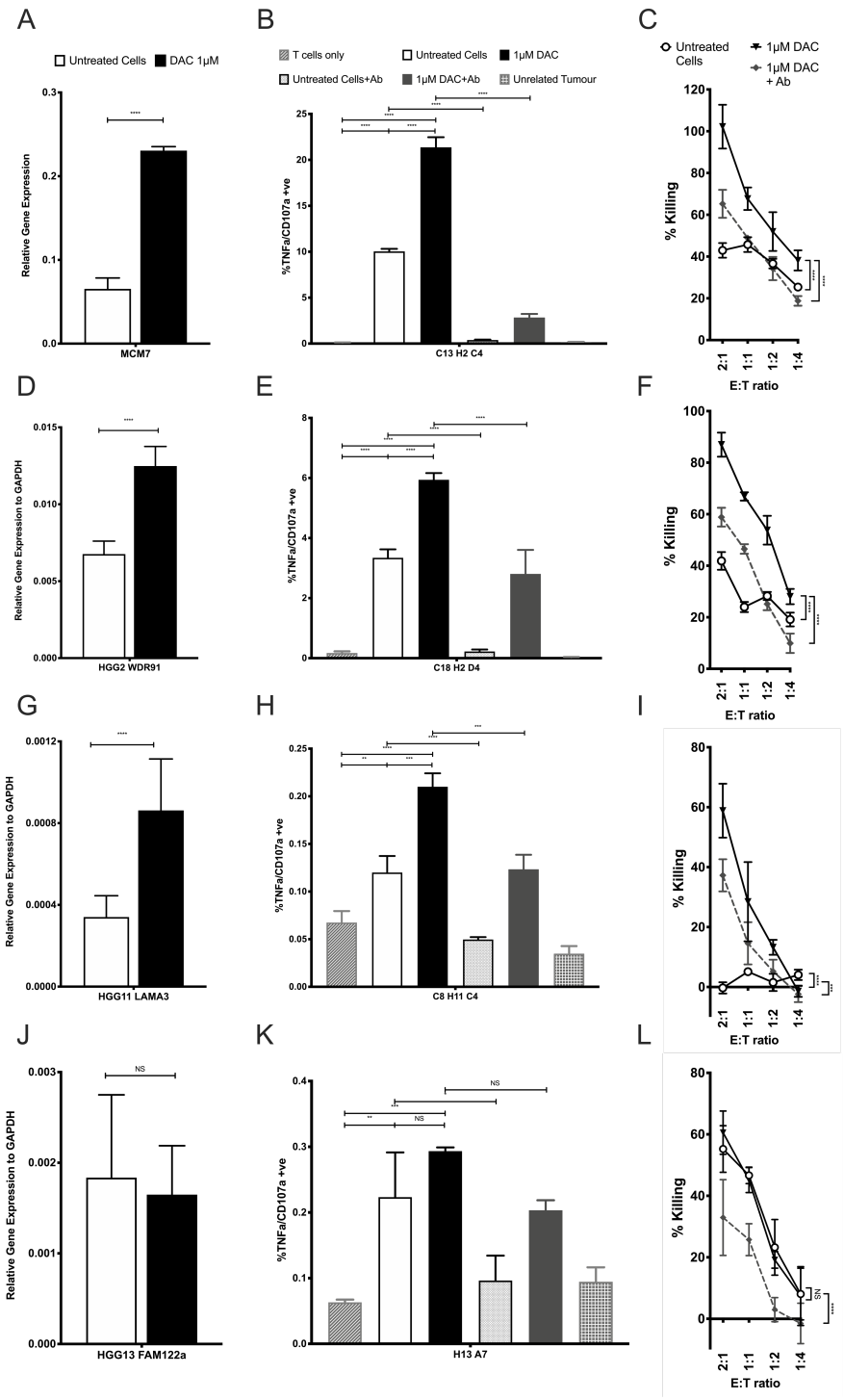


Figure 5.3 Increased expression of neoantigen leads to increased CTL activation and killing in a TCR-MHC dependent fashion. Bar plots showing relative gene expression of (A) MCM7, (D) WDR91, (G) LAMA3, (J) FAM122a to GAPDH as measured by RTqPCR for the relevant cell lines (A+D – HGG2; G – HGG11, J – HGG13). Expression is represented as $2^{-\Delta\Delta CT}$ and statistical significance as calculated by paired two-tailed student t test. Activation of (B) MCM7, (E) WDR91, (H) LAMA3 and (K) FAM122a specific CTL as measured by TNFα+/CD107a+ cells by flow cytometry. All cells pre-gated on CD3+/CD8+ population. (C) MCM7, (F) WDR91, (I) LAMA3 and (L) FAM122a specific CTL mediated killing as measured by LDH release against primary cell lines. Data shown is from technical triplicates representative of a minimum of 3 biological replicates. Statistical significance as calculated by 2-way ANOVA. Ab – W6/32 MHC class I blocking antibody.

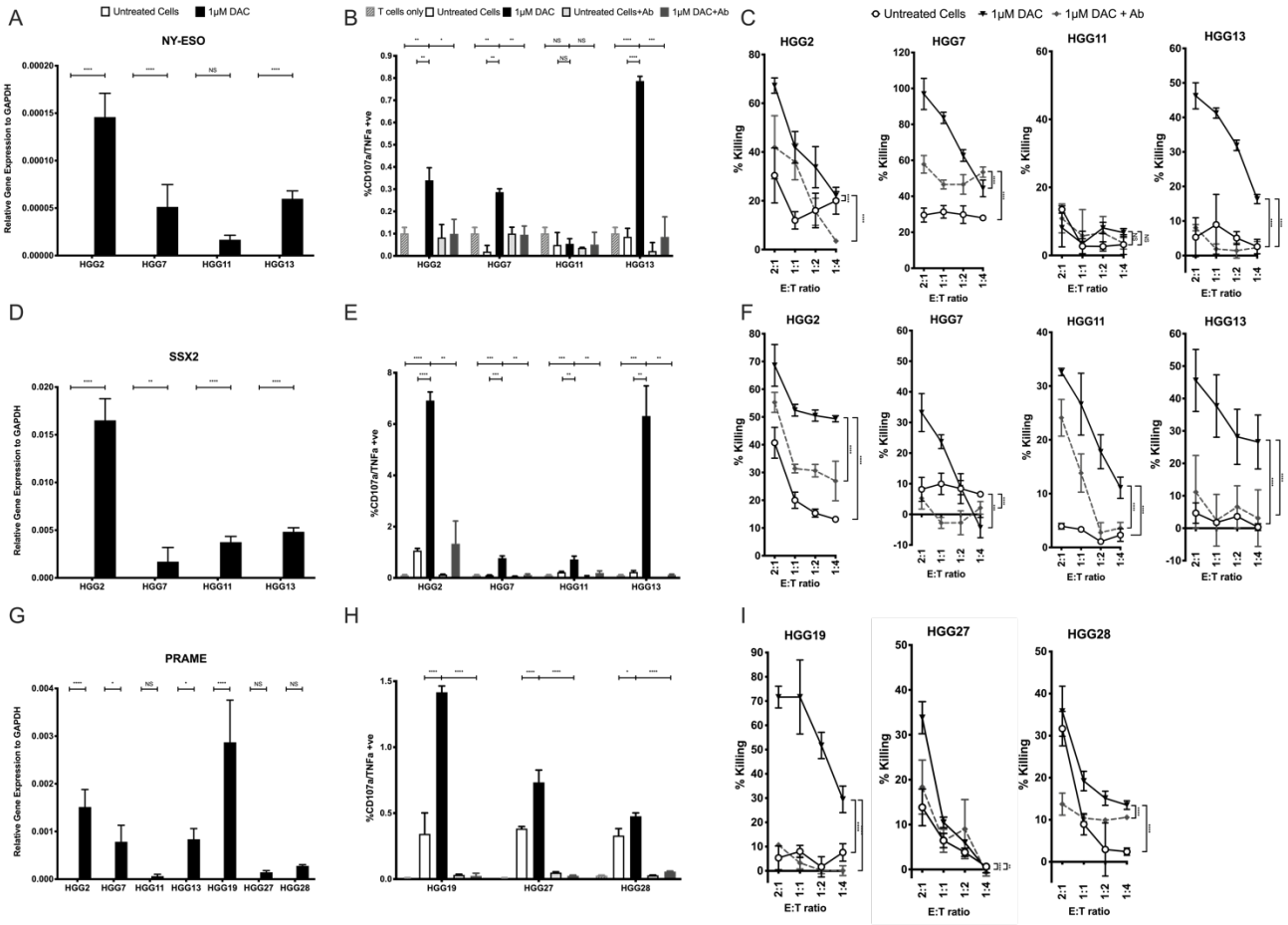


Figure 5.4 Increased expression of cancer testis antigens leads to increased CTL activation and killing in a TCR-MHC dependent fashion. Relative expression of (A) NY-ESO, (D) SSX2 and (G) PRAME to GAPDH as measured by RTqPCR. Expression is represented as $2^{-\Delta\Delta CT}$ and statistical significance as calculated by paired two-tailed student t test. Activation of (B) NY-ESO, (E) SSX2 and (H) PRAME specific CTL as measured by TNFα+/CD107a+ cells by flow cytometry. All cells pre-gated on CD3+/CD8+ population. (C) NY-ESO, (F) SSX2 and (I) PRAME specific CTL mediated killing as measured by LDH release against primary cell lines. Data shown is from technical triplicates representative of a minimum of 3 biological replicates. Statistical significance as calculated by 2-way ANOVA. Ab – W6/32 MHC class I blocking antibody.

5.2.3. Decitabine treatment of normal human astrocytes does not lead to T cell activation

NHA cell line was subjected to the same DAC treatment as the tumour cell lines. As shown in chapter 3 (figure 3.5), although unsilenced, this increase in expression of CTA in NHA following DAC treatment is not significant when compared to changes in U87MG. To test if this small increase in gene expression leads to any functional difference, NHA, which are HLA-A2+, were also co-cultured against NY-ESO and SSX2 specific CTLs. We find that, mirroring the small change in transcription following treatment with DAC, there is also no change in the levels of activation of CTL (figure 5.5).

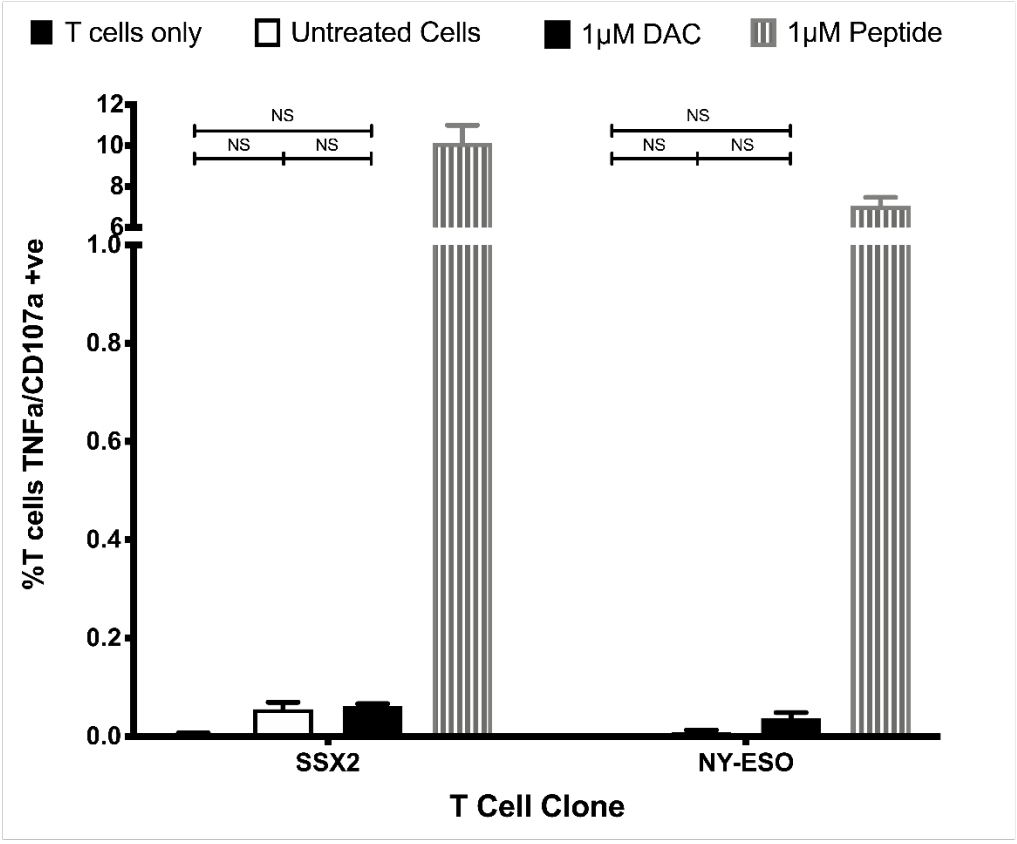


Figure 5.5 Treatment of NHA with DAC does not increase CTL recognition and activation. CTL mediated activation as measured by TNFα+/CD107a+ cells by flow cytometry. All cells pre-gated on CD3+/CD8+ population. Data shown is from technical triplicates representative of 3 biological replicates. Statistical significance as calculated by 2-way ANOVA.

5.2.4. Reporter jurkat cells transduced with specific TCR are capable of recognition of tumour with increased efficacy following decitabine treatment

Transduced reporter J8 cells with a selection of TCRs of interest show similar activation results to those from the primary CTL, with increased activation following co-culture with tumour cells treated with DAC (figure 5.6).

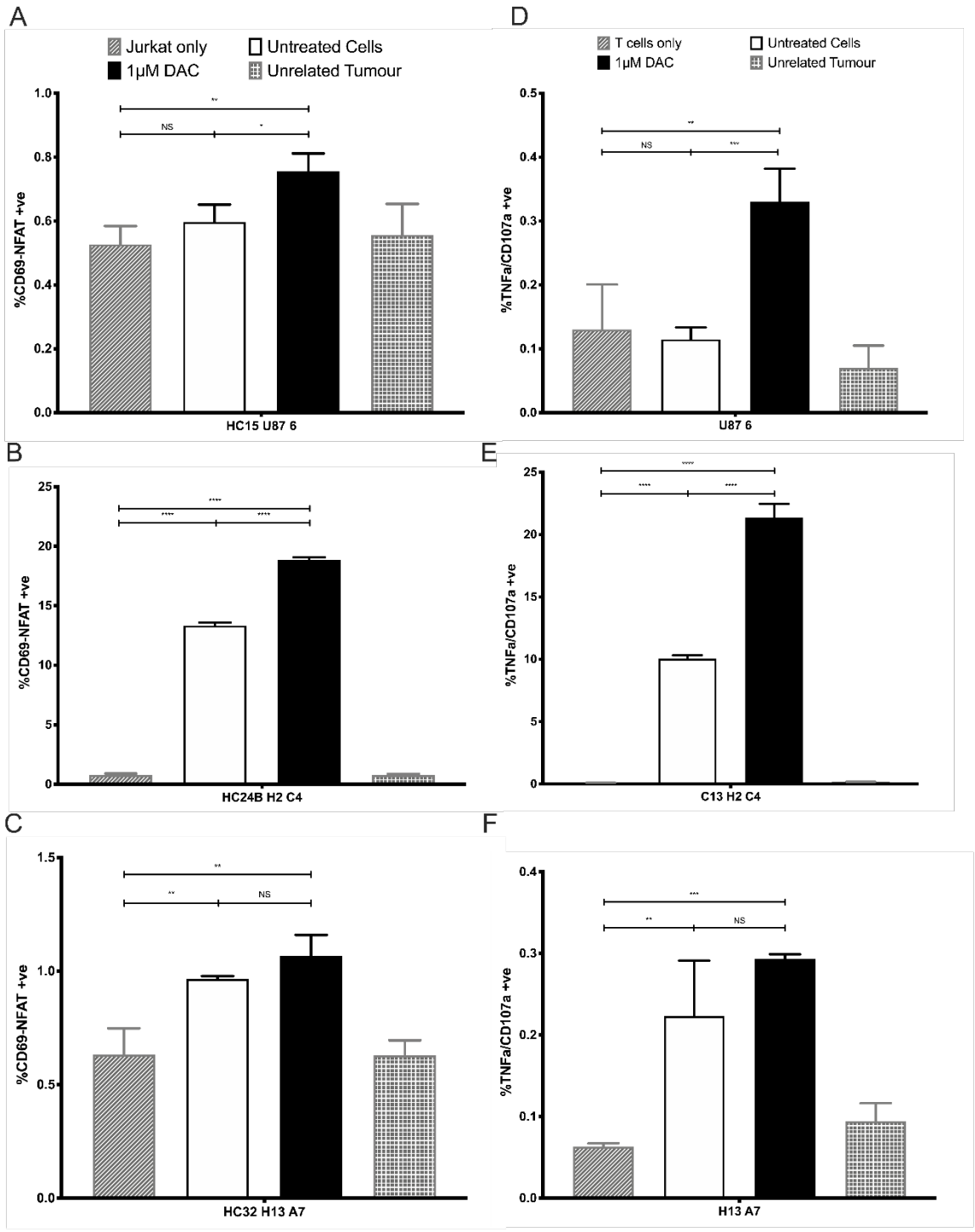


Figure 5.6 J8 reporter cells transduced with TCR show similar results to primary CTL. J8 cells transduced with relevant TCR were co-cultured with cell lines with/without DAC treatment for 24 hours and analysed for activation by flow cytometry (A,B,C). Activation as determined by NFAT-GFP+/CD69+ cells, pre-gated on mouse TCRβ+ population. Primary CTL with the same TCR co-cultures with the same cell lines are shown on the right for comparison (D,E,F). CTL activation as measured by TNFα+/CD107a+ cells by flow cytometry. All cells pre-gated on CD3+/CD8+ population. Data shown is from technical triplicates representative of a minimum of 3 biological replicates. Statistical significance calculated by 2-way ANOVA.

5.2.5. Tumour specific T cells isolated through mixed tumour lymphocyte culture show increased recognition and killing following decitabine treatment

Utilising autologous CTLs generated through MLTC, described in chapter 4, we show that some of these T cells are able to recognise tumour cells that have not been treated with decitabine in a tumour specific fashion (figure 5.7 A). For tumour reactive CTLs, treatment of the tumour cells with DAC, leads to a significant increase in activation and CTL mediated killing, again in a TCR:MHC dependent fashion (figure 5.7 B).

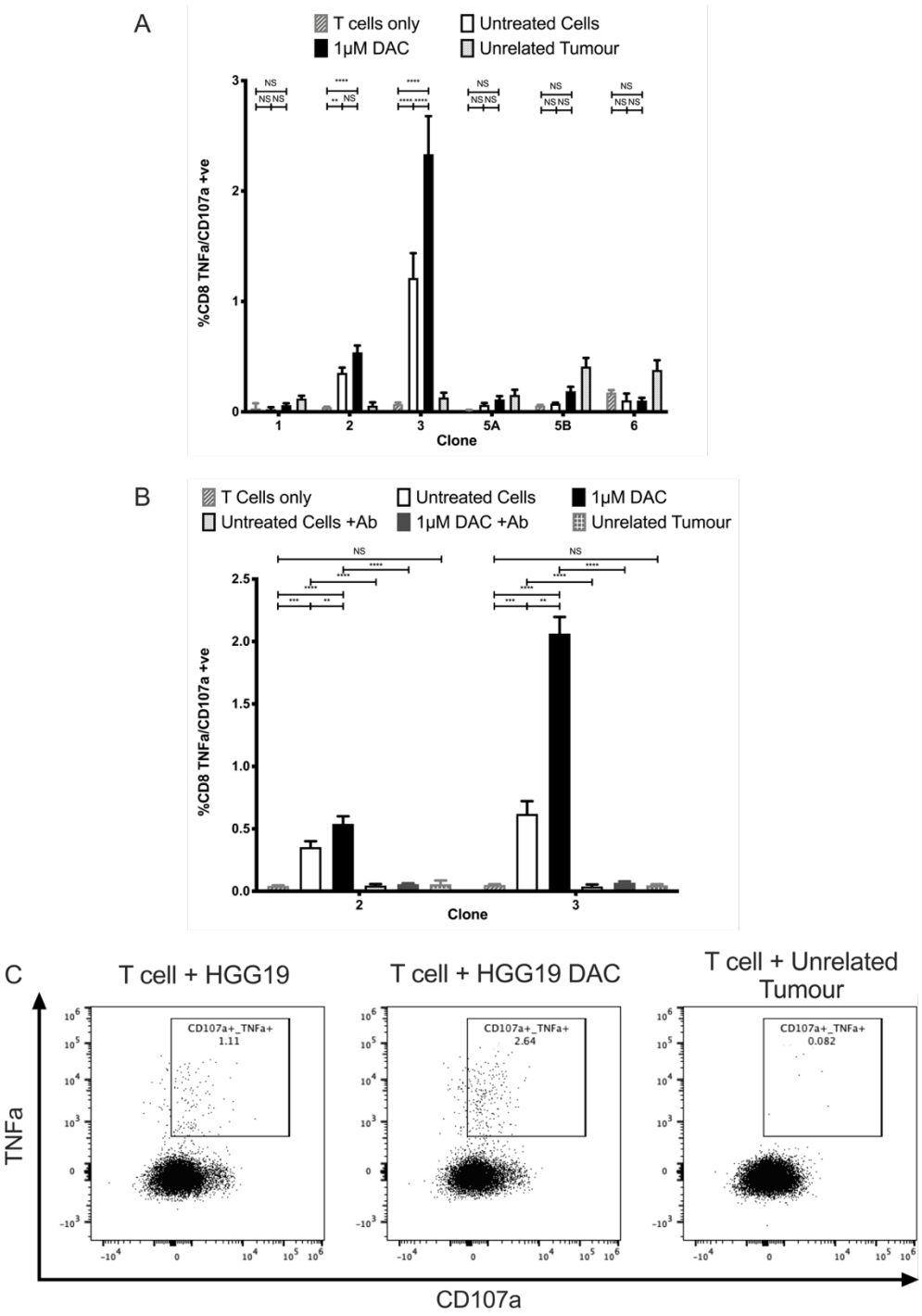


Figure 5.7 Tumour reactive T cells isolated from MLTC show increased reactivity when co-cultured will tumour cells treated with DAC. Representative bar (A, B) and dot plots (C) showing T cell activation following co-culture with tumour cells +/- DAC treatment or an HLA-matched unrelated tumour. (A) Results from the initial screen of all TCR, (B) more detailed results looking at clone 2/3 including the addition of MHC class I blocking antibody. T cell activation as measured by TNFα+/CD107a+ cells by flow cytometry. (C) Representative dot plot showing T cell activation for clone 3. All cells pre-gated on CD3+/CD8+ population. Data shown is from technical triplicates representative of a minimum of 3 biological replicates. Statistical significance calculated by 2-way ANOVA. Ab – W6/32 MHC class I blocking antibody.

5.3. Discussion

In this chapter I show that the increase in immunogenic antigens found in chapter 3 is sufficient to lead to increased T cell mediated recognition and killing in both U87MG and primary cell lines. This T cell activation and killing is significantly reduced following the addition of an MHC Class I blocking antibody (W6/32) suggesting that this effect is due to increased epitope availability on the tumour cells. We have shown earlier in chapter 3 that there is high baseline expression of MHC class I on our tumour lines and treatment with DAC does not induce any significant upregulation (figure 3.4). This would suggest that the increase in epitope availability is due to the increased expression of the genes containing mutations leading to increased antigen availability to competitively bind to MHC class I rather than an increased MHC class I availability. To further support these claims, we isolated a T cell clone that was reactive to a neoantigen produced from a mutation in FAM122a in HGG13, which was not increased following DAC treatment (figure 5.3 J). The gene is expressed in the control setting without DAC treatment and there is some activation of the neoantigen specific T cells following co-culture with untreated HGG13 tumour cells. As there is no significant increase in gene expression, there is also no increase in T cell activation and killing following treatment of the tumour cells with DAC (figure 5.3 K,L). If any increased T cell activation was due to an increase in MHC availability, it is reasonable to expect that there would be increased T cell activation and killing following DAC treatment even without increased neoantigen expression, which is not the case.

Neoantigens by their very nature, are tumour specific and so increased expression of the genes harbouring such mutations will only lead to increased neoantigen expression in the tumours specifically. However, CTA genes are present in all cells but only normally

expressed in the testis (Scanlan *et al.*, 2002). Therefore, there is the potential that DAC can also unsilenced the expression of these genes not only in tumour cells but also healthy tissue, leading to an immune response to healthy tissue. If this was the case, then this avenue of treatment would lead to unacceptably high adverse effects. To address this issue, we have shown in Chapter 3 that DAC treatment of normal human astrocytes does lead to the increase in transcripts of CTAs (figure 3.5) but this increase is not statistically significant when compared to the increase in transcripts seen in U87MG. To ensure that even this small increase in gene expression is also not functionally significant, we also tested these NHA against CTA specific T cells (figure 5.5). we show that there is no significant increase in activation of either SSX2 or NY-ESO specific T cells against NHA treated with DAC. This supports previous data published to show that DAC does not have a significant effect on CTA expression (Natsume *et al.*, 2008).

There are other non-TCR:MHC dependent routes of activation of T cells, such as through other stimulatory co-receptors, such as NKG2D (Prajapati *et al.*, 2018; Kim and Shin, 2019). Therefore, we wanted to ensure that any effect seen is due to increased TCR:MHC dependent activation. The fact that T cell activation is abrogated by an MHC class I antibody is supportive of this but to increase our confidence, we also sequenced the TCRs for our neoantigen/CTA specific T cells and transduced them into a reporter Jurkat line (J8) described in chapter 4. In figure 5.6 we show that the J8 reporter cells behave in a similar fashion to the primary T cell clones, lending further support that any effect seen is neoantigen/CTA mediated through MHC class I presentation.

In the absence of endogenous T cells responses, we have shown it is possible to generate neoantigen specific T cells from healthy donors which, along with CTA specific T cells offers

the route of adoptive transfer of such TCRs following treatment with DAC. However, this would be more time consuming and expensive and the ability to translate to a large number of patients is more limited. As mentioned in chapter 4, one limitation of this experimental design was our inability to generate autologous neoantigen specific T cells. This would enable us to show that there are neoantigen specific T cells pre-existing in patients that should be able to recognise and kill the tumour cells following treatment with DAC. To further investigate the presence of endogenous T cell responses, we utilised an MLTC approach to try and generate tumour specific T cells.

From the MLTC we were able to identify and isolate 2 CD8 TCR from the PBMC of HGG19, which when transduced into CTL, conferred reactivity against the autologous HGG19 tumour cell line (figure 5.7A). In addition, these T cells also showed increased reactivity following co-culture with the autologous tumour treated with DAC. Finally, we also show that T cell activation and killing is blocked following addition of W6/32 (figure 5.7B). Whilst a MLTC approach maximises the chances of finding tumour specific T cells, as it does not rely on any *in silico* predictions, the major limitation of utilising this method is it is not easily possible to identify the antigen that the T cell is responding to. Therefore, we cannot conclusively show that these T cells are true neoantigen or CTA specific T cells reacting to an increased expression of neoantigen as we have shown in figures 5.2 and 5.3. However, we show that these T cells react to HGG19 in a tumour specific fashion and are not activated by unrelated, HLA-matched, tumours. This, coupled with the data showing increased neoantigen expression leading to increased activation and killing by neoantigen specific T cells isolated from healthy donors, supports the notion that this increased activation of tumour specific T cells is likely to be due to increased expression of a tumour specific antigen if not neoantigen.

6. Analysis of tumour infiltrating lymphocyte repertoire

6.1. Introduction

Studies interrogating the immune infiltrate of tumours have coalesced to generate 3 broad classifications of tumours: 1)inflamed tumours with high levels of immune infiltrate, which may be exhausted following chronic stimulation; 2) immune excluded tumours where immune cells are present in the periphery but unable to infiltrate into the tumour due to various factors such as stromal inhibition or secretion of cytokines such as C-X-C motif chemokine ligand 12 (CXCL12); 3) immune desert with very little immune infiltrate due to a combination of immunological ignorance and tolerance (Chen and Mellman, 2017). GBM, unfortunately, sits in the immune desert phenotype with very few TIL, most of which, if present, are severely exhausted (Thorsson *et al.*, 2018; Woroniecka *et al.*, 2018).

It is not just the number of TIL that is relevant, it is also important to know what proportion of TIL are tumour reactive. A small number of TIL that are all tumour reactive could be more effective than a large number of non-reactive bystander TIL. It is known from adoptive cell transfer treatments that the majority of expanded TIL products produced and infused into patients are not actually tumour reactive (Kato *et al.*, 2018; Ahmadzadeh *et al.*, 2019). As these TIL are massively expanded to achieve the required numbers for therapeutic injections, there is inherent bias in this analysis, and it may be that tumour reactive TIL are more likely to be exhausted and expand less compared to their bystander counterparts. Therefore, to get a better reflection of the TCR repertoire in TIL, unbiased analysis of fresh tissue is needed. Work performed to interrogate the TCR repertoire of TIL in GBM shows that there is greater TCR diversity in tumours than in healthy brain. In

addition, divergence of the TCR repertoire in GBM, although to a lesser degree than in low grade gliomas, as compared to the periphery indicates that there is likely to be a tumour associated T cell response (Sims *et al.*, 2016). This is not dissimilar to other studies analysing TCR repertoires in other tumours (Li *et al.*, 2016; Bobisse *et al.*, 2018; Ahmadzadeh *et al.*, 2019; Joshi *et al.*, 2019). The majority of studies interrogate the TCR repertoire through bulk sequencing and could only comment on T cell clonotypes. Inferences are made that if a certain TCR clonotype is expanded, especially following treatment, then it is likely that they are tumour specific (Cloughesy *et al.*, 2019; Schalper *et al.*, 2019). More recent advances have allowed researchers to interrogate the TCR repertoire at a single cell level, allowing for full, paired, identification of TCRs. This not only allows for more accurate portrayal of the TCR diversity, but also allows researchers to test individual TCRs for tumour reactivity by transducing specific TCRs into reporter cells or primary T cells. By doing this, Scheper et al. have shown that in melanoma, which is an inflamed tumour with a high TMB, there are many tumour reactive TIL, most of which are expanded. However, in other cancers, such as ovarian carcinoma and colorectal carcinoma, which have lower TMB, the incidence of tumour reactive TIL is much lower and variable and is rarely expanded (Scheper *et al.*, 2019). This highlights the importance of treating each individual tumour type differently and it is not possible to simply infer tumour specificity from expansion alone. We believe that this is especially true for GBM, which occupies an immunologically 'distinct' niche in the brain.

In this chapter we present our data interrogating the TCR repertoire in GBM across 13 patients at a single cell level. We further interrogate the TCR repertoire for 1 patient to look for tumour specificity. Additionally, we also performed unbiased single cell *ex vivo* clonal expansion of TIL for one patient to identify tumour specific T cells.

6.2. Results

We performed scTCRseq analysis of 13 patients TIL and PBMC, including 2 patients for which we were able to obtain both a primary and recurrent sample (table 6.1).

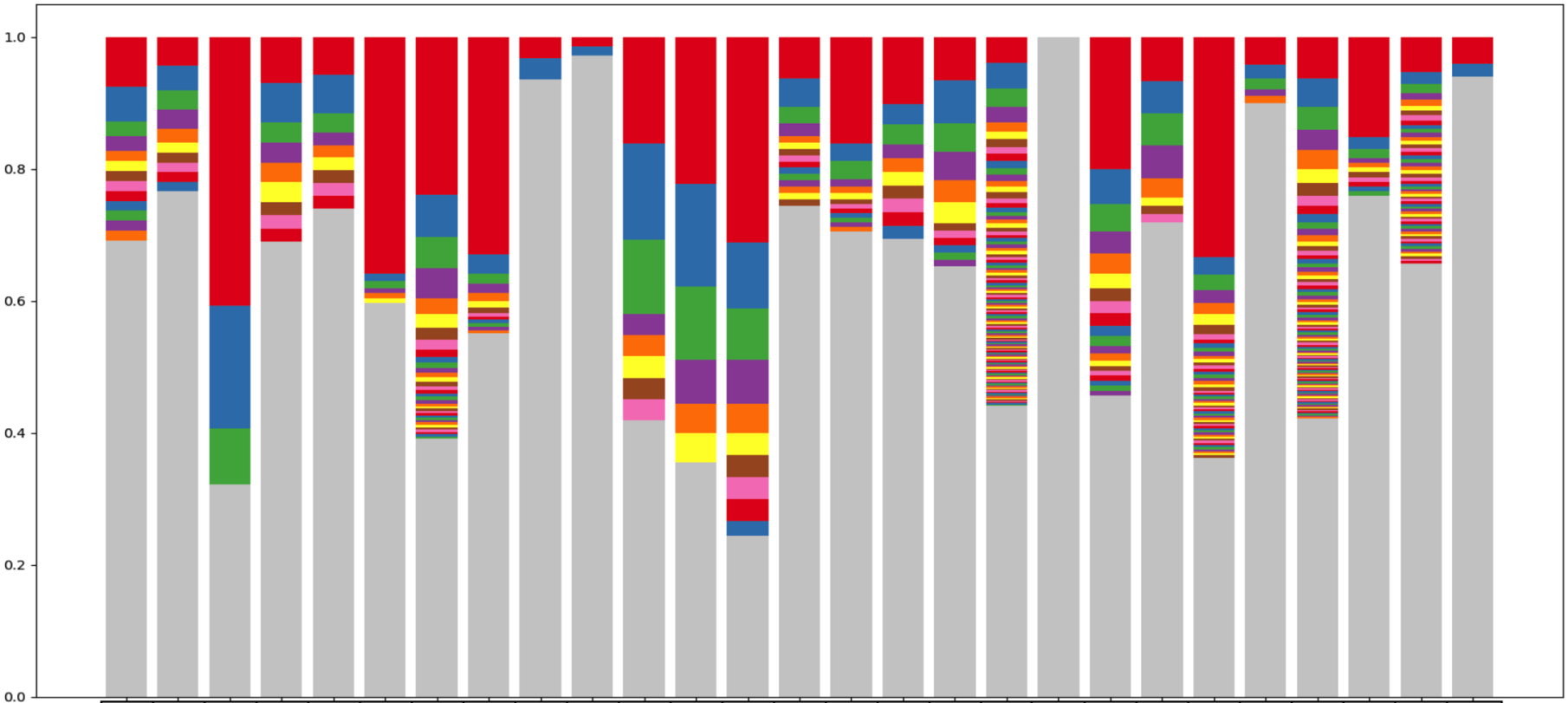
Patient	Primary/Recurrent tumour	IDH status	MGMT promoter methylation	Other
HGG5.2	Recurrent	WT	Hypermethylated	
HGG11	Primary	WT	Unmethylated	
HGG20	Primary	WT	Hypermethylated	
HGG21	Primary	WT	Hypermethylated	
HGG22.1	Primary	Mut	5-10% methylation	
HGG22.2	Recurrent	Mut		
HGG26	Primary	WT	Hypermethylated	TERT mutation
HGG27.1	Primary	WT	Hypermethylated	
HGG27.2	Recurrent	WT		
HGG29	Primary	WT	Hypermethylated	
HGG32	Primary	WT	Unmethylated	
HGG33	Primary	WT	Unmethylated	Gliosarcomatous focus
HGG35	Primary	WT	Unmethylated	
HGG36	Primary	WT	Unmethylated	Gliosarcoma variant
HGG37	Primary	WT	Unmethylated	

Table 6.1 Integrated histopathological and molecular characteristics of GBM samples. 14 samples from 12 patients were collected and analysed. The majority of GBM (12/14) were IDH1 wild-type and 6/12 GBM were hypermethylated at the MGMT promoter site. In addition, HGG26 carried a TERT C250T mutation and HGG33 and 36 had gliosarcomatous foci. WT – wild-type; Mut – mutant (R132H), hypermethylated >10% methylation, unmethylated <5% methylation

6.2.1. Tumour infiltrating lymphocyte repertoire varies between patients

The TCR repertoire varied between different patients (figure 6.1). However, there does seem to be a trend towards with increased diversity in the PBMC and increased clonality in the TIL repertoire (figure 6.1,6.2A). In addition, when we look for overlap of TCR between TIL and PBMC we see that overall, the vast majority of TCR are not shared but there are some shared TCRs generally in all patients (figure 6.2B, appendix). Finally, we compared our TCR repertoires against a public TCR specificity database, VDJdb (https://vdjdb.cdr3.net). We found that a proportion of the TCR identified in our samples,

both PBMC and TIL, were public sequences found to be reactive against both viral and tumour antigens (figure 6.2C, appendix). This was most notably the case for the most expanded TCR in HGG36 whose CDR3 alpha has been previously described to recognise the EBV antigen AVFDRKSDAK presented on HLA-A*11.



Patient ID	20	20	21	21	22	22	22	22	26	26	27	27	27	29	29	32	32	33	33	35	35	36	36	37	37	5	5
Tissue	T	P	T	P	T	P	T	P	T	P	T	T	P	T	P	T	P	T	P	T	P	T	P	T	P	T	P
Prim/Rec	P	P	P	P	P	P	R	R	P	P	R	P	P	P	P	P	P	P	P	P	P	P	P	P	P	R	R
No. cells	133	137	59	100	104	273	565	385	62	46	45	90	62	207	292	98	184	1033	22	265	164	631	190	980	278	530	100

Figure 6.1 TCR repertoire of TIL and PBMC in different patients. Stacked bar charts showing TCR repertoire of different patients' TIL and PBMC. Grey column represents singlet population, coloured bars represent TCR seen >1. Colours not indicative of similar TCR. Tissue row: T – Tumour, P – PBMC, Prim/Rec row: P – primary, R – Recurrence.

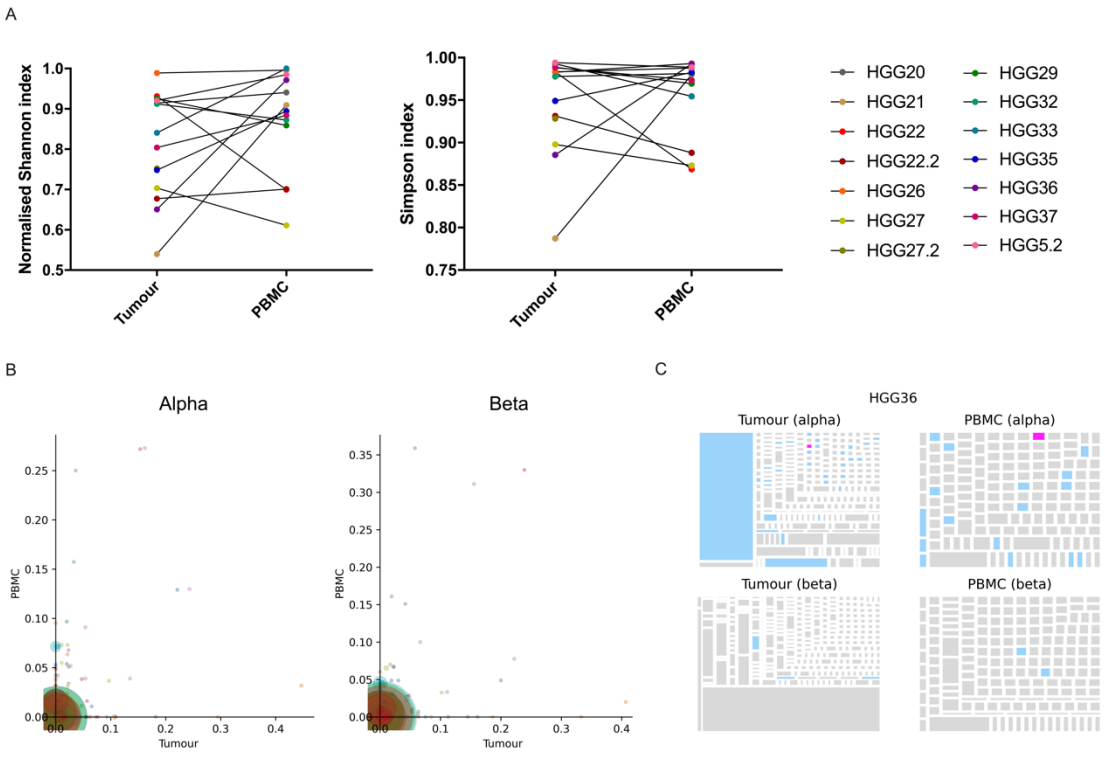


Figure 6.2 TCR repertoire comparison between different patients. (A) Plot showing normalised Shannon and Simpson indices of TIL and PBMC for individual patients. (B) Dot plot showing TCR overlap between TIL and PBMC within individual patients. X and Y axis represent the frequency of TCR in the repertoire. Size of dot represents number of unique TCR for that position. Colour of dot represents different patients. (C) Plot showing TCR repertoire in PBMC and TIL in primary and recurrent samples and identification of public TCR for HGG36. Size of bar represents the frequency of TCR in repertoire. Blue bar – public viral specific TCR, magenta – public cancer associated antigen specific TCR. Individual plots for other patients can be seen in appendix.

We performed clustering of TCRs by similarity of either their α/β chains. Using this method, we were able to identify 10 α chain clusters and 1 β chain cluster. As can be seen in figure 6.3. The majority of these clusters are patient specific, with only 1 α chain cluster seen in more than 1 patient. Of these clusters, cluster 1 on further analysis represents a classical mucosal associated invariant T cell (MAIT) cluster, described later in this chapter.

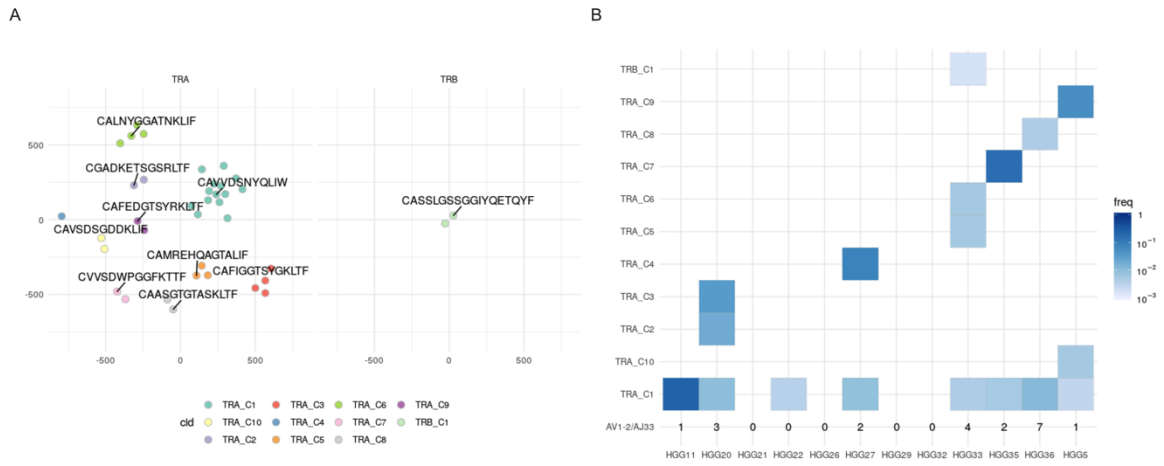


Figure 6.3 Clustering of TCR by similarity reveals distinct α/β clusters. (A) Clustering of TCRs by similarity reveals 10 distinct α clusters and 1 β cluster. Colours represent different TCR clusters and each dot represents a distinct TCR in that cluster. Labelled dot is CDR3 sequence that the cluster is based on. (B) distribution of TCR clusters between different samples. All clusters except TRA_C1 are unique to individual patient samples. TRA_C1 is the MAIT TCR cluster that is shared between 8 patients. Number on X axis represents number of TCR in TRA_C1 that have the canonical MAIT V1-2/J33 α chain.

6.2.2. Tumour infiltrating lymphocyte repertoire in primary and recurrent samples

We then compared the TCR repertoire seen in the primary samples to that taken on recurrence for HGG22 (there were unfortunately not enough cells to make any meaningful comparison for HGG27). We find that the diversity of the repertoire remains fairly similar between timepoints although there does seem to be an increase in the number of shared TCR between TIL and PBMC at recurrence (figure 6.4A). The relative proportion of the repertoire of these shared TCR is broadly similar between the TIL and PBMC. There are also TCR that are shared between the primary and recurrence samples. However, in contrast to the shared TCR between PBMC and TIL, the TCR shared between primary and recurrent samples do not make up the same proportion of the repertoire, with some TCR expanding and some contracting (figure 6.4B). Finally, similar to other samples, there are public TCR

specific to viral and cancer antigens that are present in both the primary and recurrent samples (figure 6.4C).

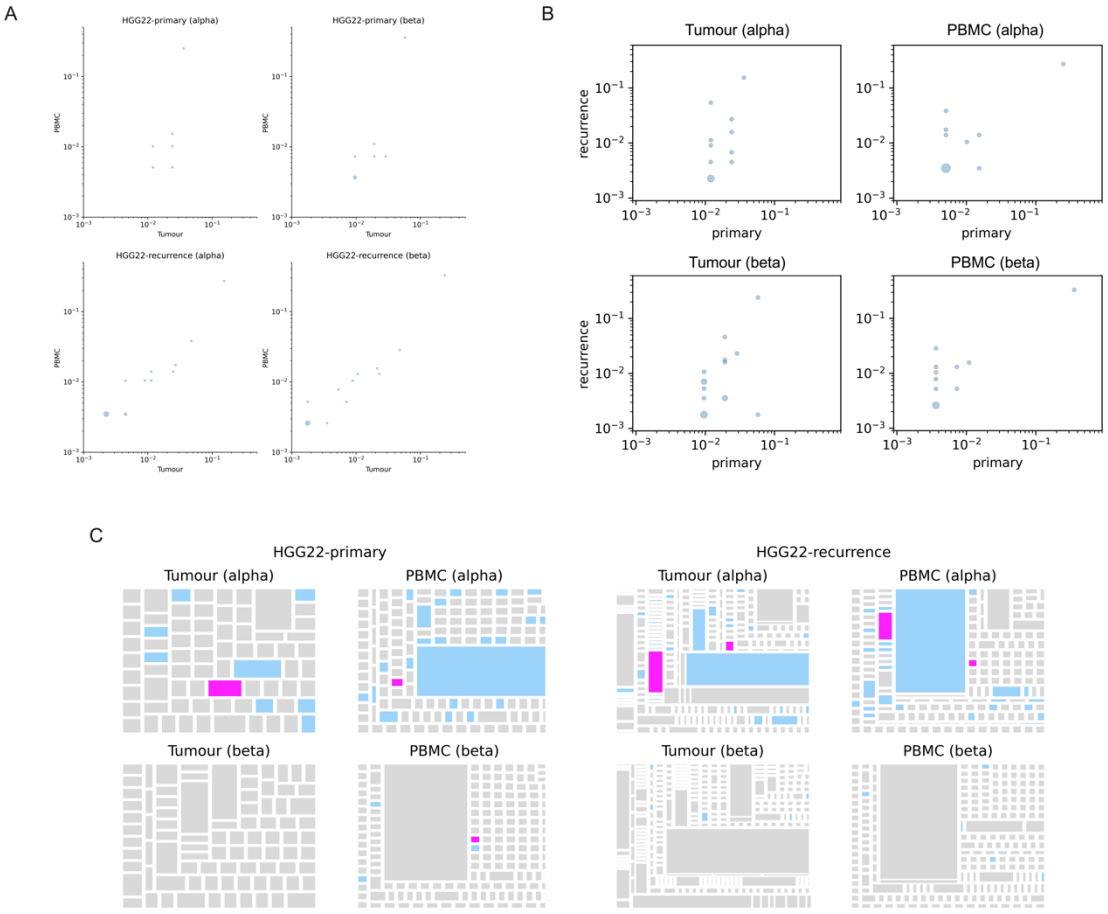


Figure 6.4 Comparison of HGG22 primary and recurrent tumour and PBMC samples. Dot plots showing TCR overlapping TCR between (A) PBMC and TIL in primary and recurrent samples and (B) primary and recurrent samples for PBMC and TIL. X and Y axis represent frequency of TCR. Size of dot represents number of unique TCR for that position. (C) Plot showing TCR repertoire in PBMC and TIL in primary and recurrent samples and identification of public TCR. Size of bar represents frequency of TCR in repertoire. Blue bar – public viral specific TCR, magenta – public cancer associated antigen specific TCR.

6.2.3. Transduction of TCR into jurkat reporter cells reveals tumour specific T cells

For 1 patient (HGG36) we transduced any clonally expanded TCRs (TCR seen >1 in either TIL or PBMC) into our reporter J8 or J4 cells, depending on whether the TCR was from a

CD8 or CD4 T cell, respectively. We then tested these reporter cells against the autologous tumour lines.

We were able to identify 2 TCRs in HGG36 that were reactive to the autologous cell line (TCR29 and TCR43). Neither TCR was one of the most expanded TCRs (figure 6.5A). On further analysis, TCR 29 was a classical MAIT TCR, which formed one of the TCRs seen in the MAIT cluster. TCR 43 was a CD4 α/β TCR, which was not part of any TCR cluster. Both TCRs showed reactivity against the autologous cell line (figure 6.5B). What was interesting is that TCR 43, despite being a CD4 α/β TCR, does not seem to be recognising a peptide presented on MHC class II. In fact, activation of TCR43 is not blocked with either MHC class I (W6/32) or II (L243 or Tu39) blocking antibodies (figure 6.5C). Furthermore, TCR 43 was also cross reactive against other tumour lines and showed increased reactivity following treatment with DAC (figure 6.5D).

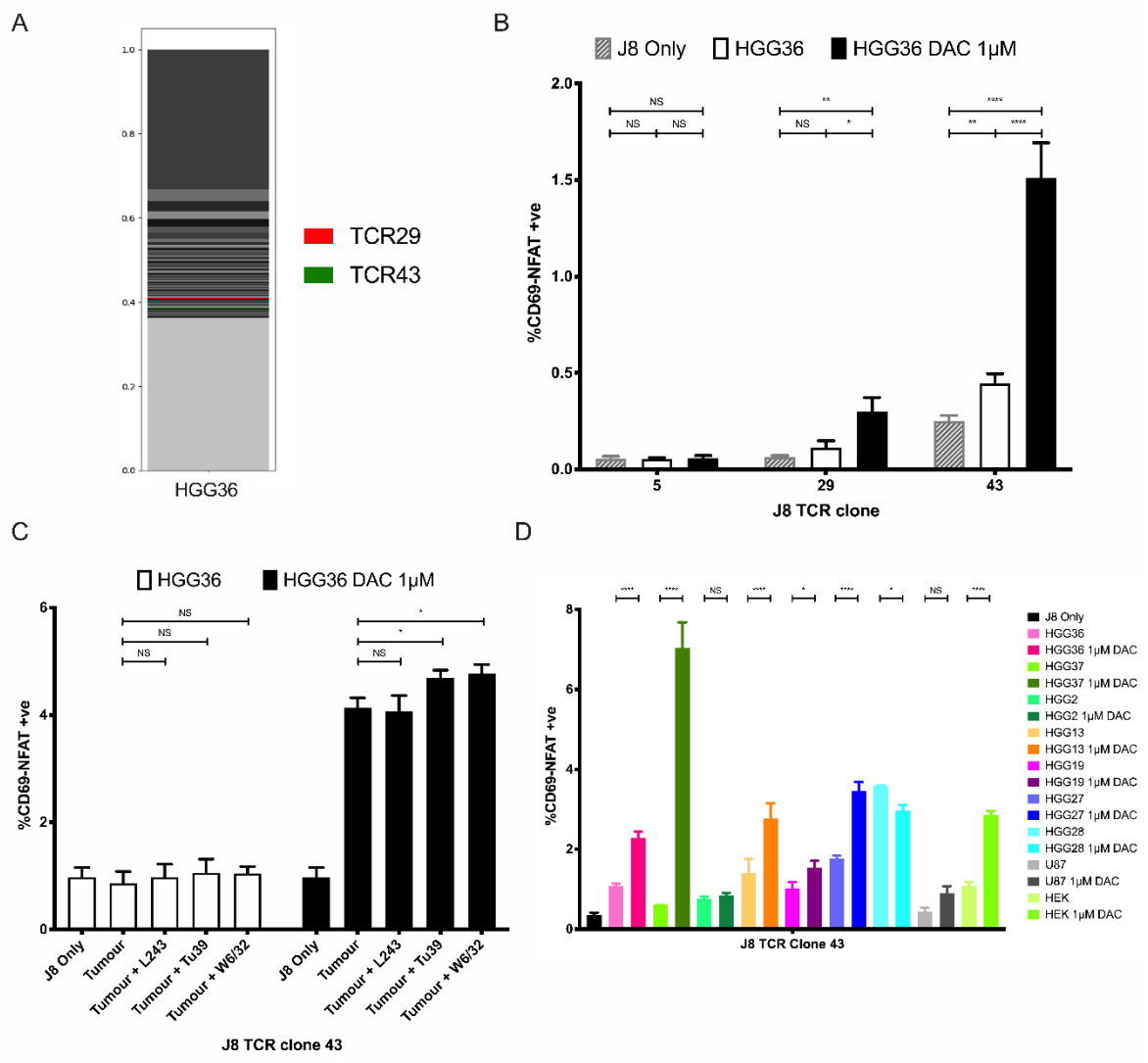


Figure 6.5 HGG36 clones 29 and 43 are tumour reactive and clone 43 shows cross reactivity to multiple GBM cell lines and increased following DAC treatment. (A) Stacked bar plot showing TCR repertoire of HGG36 and location of TCR 29 and 43 within this. (B-D) Bar plots showing activation of J8 reporter cells transduced with TCR identified from scTCRseq of HGG36 TIL and PBMC. (B) Activation of HGG36 clones 5, 29 and 43 with HGG36 +/- DAC treatment. (C) Activation of HGG36 clone 43 following co-culture with HGG36 +/- DAC in the presence of different MHC blocking antibodies. (D) Activation following co-culture with different cell lines +/- DAC treatment. Data shown is from technical triplicates representative of a minimum of 2 biological replicates. Statistical significance as calculated by paired 2-tailed student t test. control – J8 + tumour cells with no blocking antibody; L243 – HLA-DR blocking antibody, Tu39 – pan MHC class II blocking antibody; W6/32 – plan MHC class I blocking antibody.

6.2.4. MAIT TCR analysis in GBM

An interesting result from the TCR repertoire analysis was the presence of a MAIT TCR cluster in 8/13 patients (figure 6.2B). In addition, we discovered that 1 out of the 2 TCR tested for HGG36 that were tumour reactive was a MAIT TCR. To investigate this further, we tested this MAIT TCR reporter line further. Figure 6.6 shows that the MAIT TCR is activated in the presence of HGG36, especially following DAC treatment and this effect can be blocked with an anti-MR1 blocking antibody (figure 6.6A). In addition, it is not activated following co-culture with U87MG with or without pre-treatment with DAC. However, either transduction of MR1 into the U87MG cell line (U87-MR1) or pre-treatment of U87MG with 5-OP-RU in the U87MG cells leads to activation of the J8 reporter cells. Treatment of the U87MG-MR1 cell line with DAC leads to even higher levels of activation (figure 6.6B).

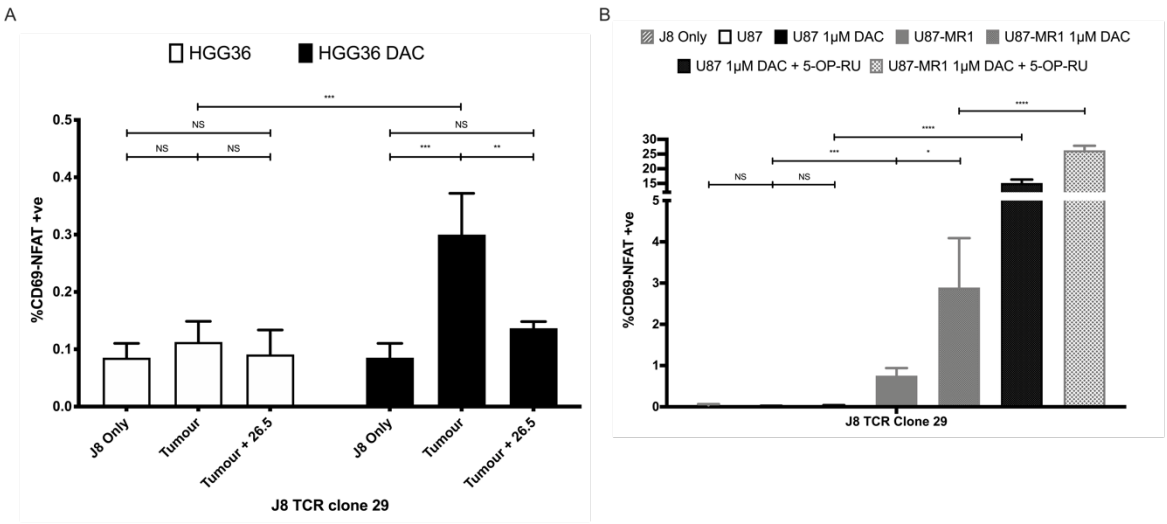


Figure 6.6 HGG36 TCR 29 is a MAIT TCR whose activation is blocked by MR1 blocking antibody and activated by MR1 transduction and 5-OP-RU. Bar plots showing activation of J8 cells transduced with HGG36 TCR 29 when co-cultured with (A) HGG36 +/-DAC +/- MR1 blocking antibody; (B) U87 +/- DAC +/- 5-OP-RU and U87-MR1 +/- DAC +/- 5-OP-RU. Data shown is from technical triplicates representative of 2 biological replicates. Statistical significance as calculated by (A) 2-way ANOVA and (B) paired 2-tailed student t test. Tumour – J8 co-cultured with tumour cells without blocking antibody; 26.5 – anti-MR1 blocking antibody.

Furthermore, we show that this MAIT TCR is activated across a wide range of tumours and treatment of the different cell lines with DAC has a variable effect on the activation of the reporter cells, which is related to the MR1 expression (figure 6.7).

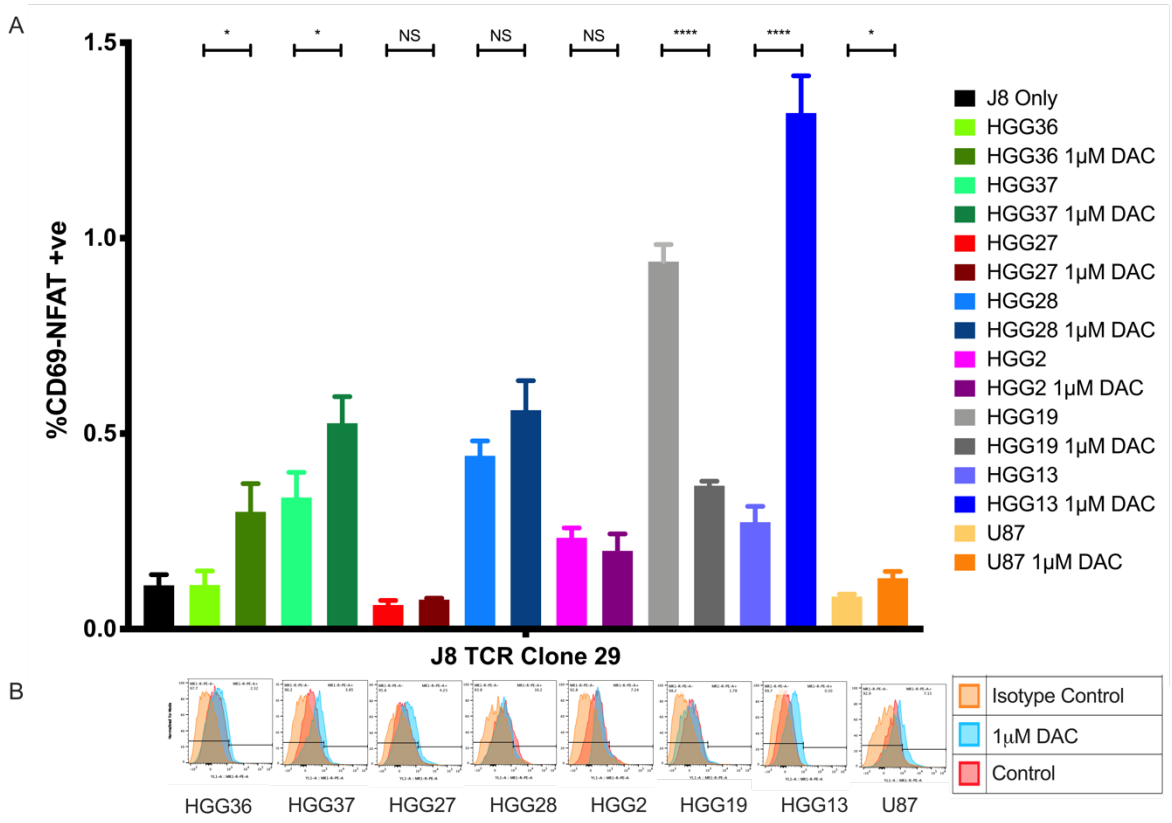


Figure 6.7 HGG36 TCR29 is activated by a range of different GBM cell lines and is related to MR1 expression. (A) Bar plot showing activation of J8 cells transduced with HGG36 TCR 29 when co-cultured with a range of GBM cell lines. (B) Histogram plots showing MR1 expression on the respective cell lines. Data shown is from technical triplicates representative of 2 biological replicates. Statistical significance as calculated by paired 2-tailed student t test.

6.2.5. Ex vivo single cell clonal expansion of tumour infiltrating lymphocytes and PBMC allows for identification of tumour specific TCR

In addition to performing scTCRseq, we also performed single cell *ex vivo* clonal expansion, using PHA, of the TCRβ+ fraction of the TIL and either the TCRβ+/CD39+/HLADR+;

TCRβ+/CD103+ or TCRβ+/HLADR+/PD1+ fraction of the PBMC for 1 patient (HGG37). Utilising this technique, we were able to expand 18/223 clones from the TIL and 26/314 from the PBMC. We then screened these clones against the autologous cell line with and without DAC treatment and found 5/18 TIL and 10/26 PBMC clones to be potentially tumour reactive (figure 6.8). Of the potentially tumour reactive clones from the TILs, all 5 were HLA-DR+/PD1+ and 4/5 were also CD39+. Of the 10 PBMC expanded clones that were tumour reactive, 5 were originally HLADR+/CD39+ and 5 were CD103+. Of the 5 CD103+ clones, 2 were also CD39+ and 1/5 HLADR+/CD39+ reactive clones was also CD103+.

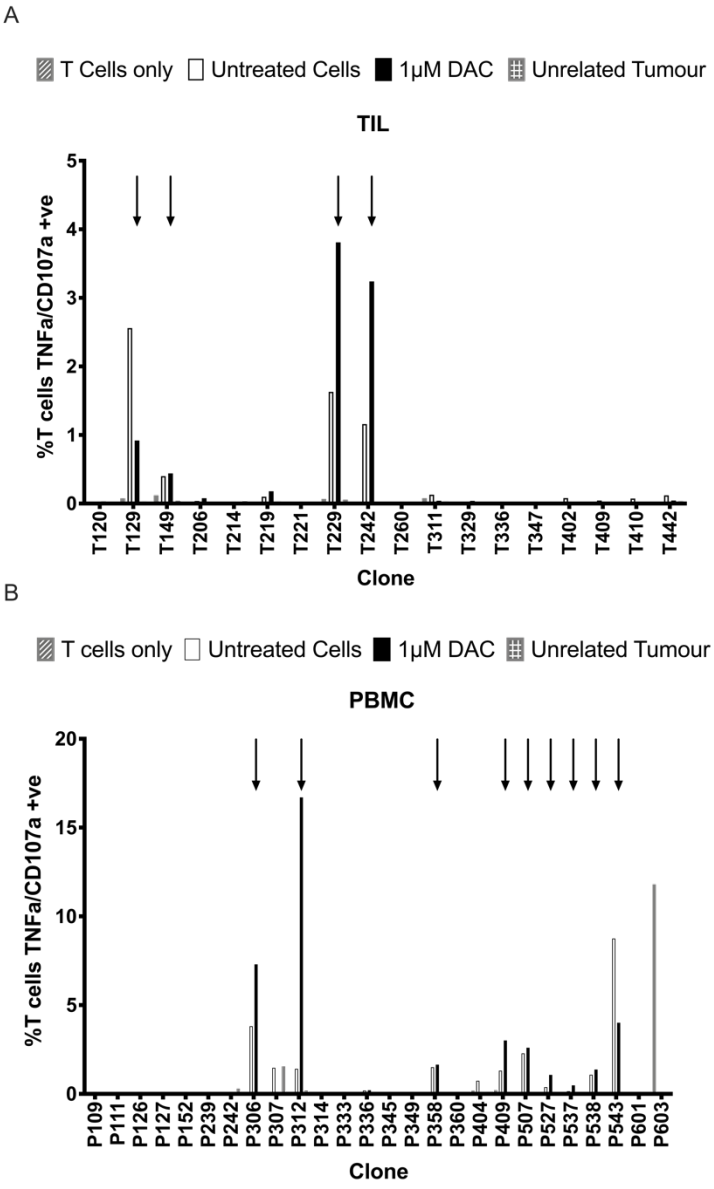


Figure 6.8 *Ex vivo* expanded tumour reactive T cells can be isolated from both TIL and PBMC. Bar plots showing activation of *ex vivo* expanded T cell clones. Expanded T cell clones were co-cultured against tumour cells. T cell activation as measured by TNFα+/CD107a+ cells by flow cytometry. Arrows indicate T cell clones that showed reactivity against HGG37 cell line. Cells pre-gated on CD3+ and CD4+ or CD8+ population as appropriate.

Unfortunately, not all of the clones, especially the tumour reactive ones, were able to be expanded for further testing. Of the cell lines that did expand further, we performed further replicates and were able to confirm that they were tumour specific and

interestingly 4/6 T cell clones also showed increased activation when co-cultured with tumour cells treated with DAC (figure 6.9).

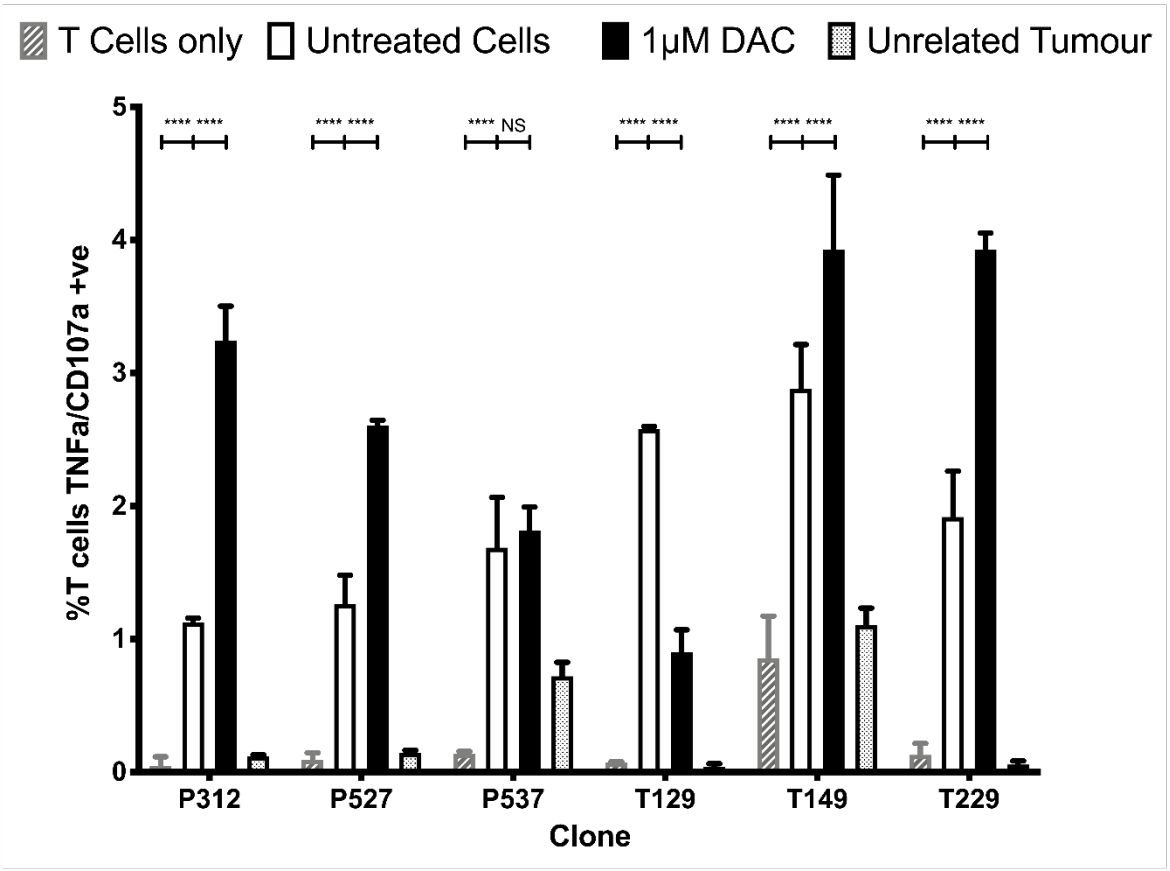


Figure 6.9 Some *ex vivo* expanded tumour reactive T cells also show increased activation following co-culture with tumour cells treated with DAC. Bar plots showing activation of T cells. Tumour reactive T cell clones were co-cultured against tumour cells with and without DAC treatment and irrelevant HLA-match tumour. T cell activation as measured by TNFα/CD107a+ve cells by flow cytometry. 4/6 T cell clones show increased reactivity following co-culture with DAC treated cell line, 1 T cell clone shows unchanged activation and 1 T cell clone shows reduced activation. Cells pre-gated on CD3+/CD4+ population. Statistical significance calculated by 2-way ANOVA.

We also sequenced the TCR of the expanded clones and compared them with scTCRseq data acquired directly *ex vivo*. We were able to identify 8 expanded T cell clones with 6 unique TCR sequences that were also present in the scTCR repertoire (table 6.2). Out of these TCR, 3/6 were tumour reactive and interestingly, it seems that the tumour reactive

TCR were not clonally expanded and only seen once in the scTCR repertoire analysed. This again supports the data seen for HGG36, where the tumour reactive TCR are not the most clonally expanded TCR.

Clone	Frequency in TIL	Frequency in PBMC	Tumour Reactive
P306	1	0	Yes
P314*	12	5	No
P345*	12	5	No
T120*	12	5	No
T219	0	1	Yes
T242	1	0	Yes
T258	1	0	-
T410	2	0	No

Table 6.2 Tumour reactive clones from *ex vivo* expansion that are also seen in scTCRseq repertoire. Expanded clones were sequenced and compared to the single cell TCR sequencing data. Frequency in TIL – number of times TCR was seen in the TIL scTCRseq repertoire; frequency in PBMC – number of times TCR was seen in PBMC scTCRseq repertoire. No reactivity data shown for T258 due to poor expansion of T cells. *represents clones with the same TCR

6.3. Discussion

The majority of studies interrogate the TCR repertoire through bulk sequencing and are only able to comment on T cell clonotypes and therefore unable to test for tumour specificity. Here, we have explored the TCR repertoire of GBM at a single cell level. We show that the TCR repertoire varies between different patients, with differing levels of diversity with a general trend of increased diversity in the PBMC as compared to the TIL. On further comparison of the PBMC and TIL TCR repertoires within patients, we show that the vast majority of TCRs are not shared between both compartments, similar to other studies looking at bulk TCR sequences in GBM (Sims *et al.*, 2016). The authors concluded that the TCR repertoire divergence seen represented an enrichment for tumour specific TCR within the TIL fraction. Indeed, many papers analysing TCR diversity and clonality make

the assumption that the clonally expanded TCR in the TIL are tumour reactive (Cloughesy *et al.*, 2019; Schalper *et al.*, 2019). Whilst this has been shown to be the case in some tumours such as non-small cell lung cancer (Joshi *et al.*, 2019), we know that this is not true for all cancers (Simoni *et al.*, 2018; Scheper *et al.*, 2019). Here, we show that this is not the case in GBM. By comparing the TCR repertoire to that of a public database of known TCR reactivity (VDJdb), we find that a significant proportion of the most expanded TCR are public and specific to viruses (figure 6.1, 6.3, appendix). This could represent non-specific T cells that are attracted to and trapped in an inflamed microenvironment. In addition, we also find public cancer related antigen specific TCR, which could represent a small cohort of tumour specific T cells. However, this data should be interpreted with some caution as the matching was based upon single chains as opposed to paired α/β TCR and it may be that any individual chain matched could confer a different specificity when paired differently. In addition, we were much more successful in matching TCR α chains compared to TCR β chains, reflecting the bias in the database for α chains. Nevertheless, despite these caveats it does seem that the most expanded clones are not tumour specific. We were also able to compare the TCR repertoire of HGG22 primary and recurrent samples. Here we show that the TCR diversity is relatively similar between the 2 timepoints although the relative frequency of some shared clones does change with time. Further work to transduce these TCR of interest is ongoing and will hopefully provide insight into TCR dynamics over time.

We also show that it is possible to utilise the cDNA product from our scTCRseq libraries as a template to transduce the TCR into our J8 reporter system (described in chapter 4). This

allows a semi high-throughput method of testing TCR of interest for tumour specificity. We have performed this on 1 patient (HGG36) and successfully managed to isolate 2 tumour specific TCR, of which 1 was a MAIT TCR (TCR29) and the other a CD4 α/β TCR (TCR43). What is interesting from the results of our TCR screen is that these two TCR, although seen more than once, were not greatly expanded in the repertoire (figure 6.4A) again lending support to the finding that the expanded TCR are not necessarily tumour specific.

The MAIT TCR motif was seen in 8/13 patients analysed and on further testing, we show that MAIT TCR activation is linked to MR1 expression on the tumour cells, which is also increased by DAC treatment of some of the tumour cells. Further investigations are required, but if this were a true effect then this could potentially lead to a new line of immunotherapy that was applicable across multiple patients.

TCR 43 was isolated from a CD4 T cell, which could recognise peptides presented on MHC class II. However, activation of J8 transduced with TCR43 was not blocked by either an HLA-DR (L243) or pan-MHC class II (Tu39) blocking antibody. It was not blocked by a MHC class I (W6/32) blocking antibody either, suggesting activation of this TCR is not through a classical TCR:MHC pathway. Furthermore, this TCR is also cross-reactive across multiple different primary GBM cell lines, with varying HLA haplotypes, and shows greater activation following co-culture with tumour cells treated with DAC. As yet, we have not been able to identify what the TCR is binding to but further work to try to identify this is on-going.

Finally, we also performed single cell *ex vivo* clonal expansion of T cells to test for tumour specificity. Here, we show that we were able to generate tumour specific T cell clones from both TIL and PBMC utilising this method. However, the ability of some of these T cells to

expand following stimulation was limited and this was especially pronounced for the tumour reactive T cells. This is an interesting finding for 2 reasons: 1 – this could reflect a more exhausted phenotype which is less likely to expand, which could explain why majority of expanded TIL products produced and infused into patients are not actually tumour reactive (Kato *et al.*, 2018; Ahmadzadeh *et al.*, 2019); 2 – this suggests that there could potentially be many more tumour reactive T cells present that we are unable to identify using this method as the cells do not expand for testing. On matching the TCR sequences of the *ex vivo* expanded clones with the scTCRseq repertoire, we find 6 unique TCRs that are seen in both datasets. Of these, 3 were tumour reactive but were only seen once in the scTCRseq repertoire. This again supports the notion that the tumour-specific TCR are not the most expanded in TIL. Again, transduction of the clonally expanded TCR is ongoing and it will be interesting to see if we are able to identify any more tumour specific TCR with this method and whether they are highly expanded or not.

In this chapter I have shown that the TCR repertoire in GBM varies between patients and there are no clear overall patterns in general that can be seen. However, we have been able to show that a significant number of the most expanded TCR are not tumour specific and likely to be viral specific TCR that are potentially trapped in the tumour microenvironment. We also show that we are able to utilise the cDNA from our scTCRseq library to transduce TCRs of interest to test for tumour specificity in a semi high-throughput fashion and are able to identify tumour specific TCR. We are also able to identify tumour specific T cells using an *ex vivo* single-cell clonal expansion method, although the tumour specific T cells were the least successful at prolonged expansion. All of these tumour

specific TCR identified from both methods are not the most expanded TCR. There are, however, some limitations to our model in that we look for tumour reactivity against the derived primary cell line. As shown in chapter 3, although there is a large mutational overlap between the cell line and the tumour, the cell line does not completely represent the original tumour. Indeed, it is likely that, due to the heterogeneous nature of GBM, the original tumour sample that was sequenced is unlikely to include all tumour subclones and thus represent the entirety of the mutational landscape in the patient's GBM. Therefore, some of the TCRs screened may be responsive to the original tumour but not the cell line.

Overall, our results, in line with other evidence seen in other cancers (Simoni *et al.*, 2018; Scheper *et al.*, 2019), suggest that choosing the most clonally expanded TCR or expanding TIL in bulk is not an efficient method for derivation of tumour specific T cells for adoptive cell transfer in GBM. Further work to try to characterise a distinct molecular signature of tumour specific T cells could greatly improve success of identifying tumour reactive TCR.

7. Concluding remarks

7.1. Conclusions

GBM still represents a problematic cancer to treat. Despite much effort, and a plethora of clinical trials, there still remains a lack of effective treatment options. Current gold standard therapy remains gross total resection following by adjuvant chemo-radiotherapy followed by maintenance chemotherapy (Stupp *et al.*, 2005), conferring a median overall survival of only 14-24 months and a five-year survival of approximately 10% (Stupp *et al.*, 2005; Stummer *et al.*, 2012). There have been many advances in the field of immunotherapy for the treatment of cancers, especially the use of check-point inhibitors, which have revolutionised the management of previously untreatable cancers such as metastatic melanoma (Wolchok *et al.*, 2013; Topalian *et al.*, 2014; Larkin *et al.*, 2015; Robert *et al.*, 2015; Schadendorf *et al.*, 2015), non-small cell lung cancer (Borghaei *et al.*, 2015; Brahmer *et al.*, 2015; Garon *et al.*, 2015; Rizvi *et al.*, 2015; Herbst *et al.*, 2016; Reck *et al.*, 2016) and renal carcinoma (Motzer, Escudier, *et al.*, 2015; Motzer, Rini, *et al.*, 2015). Unfortunately, this success in other cancers has not been replicated for the treatment of GBM, with trials showing limited effect on overall survival (Omuro *et al.*, 2017). This is thought to be, in part, due to the relatively low tumour mutational and neoantigen burden in GBM (Alexandrov *et al.*, 2013; Yarchoan, Hopkins and Jaffee, 2017).

In this thesis I have explored the hypothesis that there are potential immunogenic antigens that are not/lowly expressed. It may be possible to increase the immunogenicity of these immunologically 'cold' tumours by increasing the expression of neoantigens and cancer testis antigens, potentially making them more susceptible to immunotherapies. I have

shown that it is possible to increase the expression of both neoantigens and also a wide spectrum of CTA by treatment of both commercial and primary GBM cell lines with 1μM DAC. This increase in expression of immunogenic antigens in the tumour cells is sufficient to lead to an increase in antigen-specific CTL activation and killing. I have further shown that this phenomenon is also true for some autologous tumour specific T cells obtained from patients using various techniques.

In addition, in this thesis I have also explored the TCR repertoire of TIL at a single cell level to assess clonality and also tumour reactivity compared to the peripheral blood. I have shown that there is a trend towards decreased diversity and increased clonality within TIL compared to PBMC. However, the majority of the most expanded clones are not tumour reactive and are more likely to be public TCR reactive to viral antigens. The tumour reactive clones identified are more likely to less expanded and exhibit much lower potential for *in vitro* expansion.

Finally, we have also tested the reactivity of the expanded TCRs in 1 patient against its autologous cell line and identified 2 TCRs that are reactive against the autologous cell line. We show, that similar to other poorly immunogenic tumours such as ovarian cancer (Scheper *et al.*, 2019), the tumour reactive TCRs are not the most expanded TCRs in the TIL. This obviously poses further questions as to the best method for identifying tumour reactive TCRs for use in adoptive cell transfer therapies.

7.2. Clinical translation and future directions

In this thesis I have produced *in vitro* pre-clinical data to support the hypothesis that DAC treatment of GBM can increase the immunogenicity of these tumours by increased immunogenic antigen expression. I believe that the data in this thesis support the use of DAC in combination with a wide range of immunotherapeutic strategies. By increasing expression of neoantigens, DAC may potentiate the effect of checkpoint inhibitors, whose efficacy is known to be linked to the neoantigen burden (Mcgranahan *et al.*, 2016; Yarchoan *et al.*, 2017). In addition, the reproducible increase in expression of a wide range of CTA across different patients allows for the possibility of DAC treatment combined with CTA targeted immunotherapies such as vaccination, adoptive cell transfer or even bispecific antibody therapies. The advantages of targeting CTA is that these are unaltered genes and so any therapy created can be given to many/all patients with minimal alterations, leading to an 'off-the-shelf' therapeutic similar to checkpoint inhibitors. Furthermore, the fact that DAC increases a wide range of CTA means there are multiple potential antigens to target, minimising the possibility of immune escape. Finally, it may be possible to offer more tailored specific neoantigen therapies by predicting neoantigens and assessing which ones are increased following DAC treatment and utilising *in vitro* priming of either patient or healthy donor PBMC to generate CTLs specific to these neoantigens for adoptive cell transfer. This final modality, whilst being truly personalised therapy, is not realistically feasible on a large scale with cost and time being significant barriers to implementation, especially in a publicly funded health service such as the NHS. Therefore,

I believe the best possible use for our findings would be DAC combined with checkpoint inhibitors or DAC combined with a pan CTA targeted approach.

To further explore these effects the next steps we are undertaking are to model *in vivo* with an animal model. We plan to engraft our primary GBM cell lines into NOD SCID gamma (NSG) mice followed by DAC treatment and adoptive cell transfer of the neoantigen specific CTLs. We also hope to test whether the addition of a checkpoint inhibitor will confer additional survival advantage. The ultimate goal will be to translate these preclinical findings into an early phase clinical trial with a view to performing additional experiments, utilising the techniques we have optimised for these experiments, to explore changes in neoantigen expression in the tumours following treatment with DAC and any subsequent changes in the TCR repertoire and phenotype of the TIL.

In addition, we have generated a library of neoantigen specific CTLs against all 4 patient cell lines. This, along with our Jurkat reporter cell lines, we have a system to test a range of different epigenetic modulating drugs, such as HDACi or other more novel compounds as a potential drug discovery platform.

Our data interrogating the TCR repertoire in GBM shows that the most clonally expanded TCR are unlikely to be tumour reactive and tumour reactive cells are less likely to be clonally expanded. This suggests that the current techniques used for identifying tumour reactive T cells/TCR in other cancers are not easily applicable for GBM. Although we have established a semi high-throughput method for scTCRseq and testing of the TCR against autologous cell lines, the whole process is very labour intensive and takes time and would not be applicable on a large scale. Therefore, the ability to identify a distinct molecular

signature for tumour reactive T cells would be of great utility. To achieve this we aim to perform RNA sequencing using the Smart-seq 2 technique on our tumour reactive T cells identified by our screening mechanism and compare that to viral specific and other T cells of unknown specificity in the TIL.

We have also identified that MAIT cells are present in the tumour in the majority of patients. This is an interesting avenue to explore the functional relevance of this finding to see if the presence of MAIT cells in GBM is a prognostic factor, whether they are better at homing into the brain and if they are cytotoxic against these tumours *in vivo*. Should this be the case, given the common MR1 restriction of such cells, it could also lead to more 'off-the-shelf' treatment strategies for patients. Alternatively, if it is found that MAIT cells traffic better to the brain, it may be possible to utilise these cells for adoptive cell therapies instead of conventional T cells.

Finally, we have also identified a TCR from a CD4 T cell in one patient that is cross reactive against a range of primary GBM, which also shows increased activity following treatment of the tumour cells with DAC. This was an interesting and unexpected finding as the TCR does not seem to be restricted to MHC class II, as would be expected, or class I or MR1. This obviously warrants further exploration to determine the epitope and whether this can also be used as a potential pan-GBM treatment modality in combination with DAC.

8. References

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9. Appendix

9.1. HLA typing of patients

Patient	A1	A2	B1	B2	Cw1	Cw2
HGG2	02:01	24:02	18:01	44:02	05:01/03	12:03
HGG7	02:01	02:01	40:01	40:01	03:04	03:04
HGG11	02:01	68:02	44:02	53:01	04:01	05:01/03
HGG13	01:01/04N	02:01	07:02	08:01	07:01	07:02
HGG19	02:01	03:01	07:02	52:01	07:02	12:02
HGG20	02:01	24:02	07:02	07:02	07:02	07:02
HGG21	25:01	26:01	18:01	44:02	05:01/03	12:03
HGG22	02:01	26:01	15:01	44:02	03:03	05:01/03
HGG26	31:01	68:01	08:01	40:01	03:04	07:01
HGG27	03:01	68:01	07:02	15:01	04:01	07:02
HGG28	03:01	32:01	07:02	44:03	07:02	16:01
HGG29	03:01	24:02	37:01	44:02	05:01/03	06:02
HGG5	01:01/04N	01:01/04N	07:02	07:02	07:02	07:02
HGG33	02:01	31:01	07:02	35:02	04:01	07:02
HGG36	02:01	24:02	07:02	13:02	06:02	07:02
HGG37	01:01	01:01	40:02	40:02	07:01	07:01
U87	02:01	02:01	44:02	44:02	05:01/03	05:01/03

9.2. TCRseq primers and cycling conditions

Primers:

Oligo DT	AAG CAG TGG TAT CAA CGC AGA GTA CTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TVN
Alpha Chain Reverse 1	GTC TAG CAC AGT TTT GTC
Beta Chain Reverse 1	GTA TCT GGA GTC ATT GA
Template switching oligonucleotide	AAG CAG TGG TAT CAA CGC AGA GTA CAT rGrGrG
ISPCR forward	AAG CAG TGG TAT CAA CGC AGA GT
first PCR forward	**Well indexes**
Alpha Chain Reverse 2	TAC ACG GCA GGG TCA GGG T
Beta Chain Reverse 2	GTA TCT GGA GTC ATT GA
Second PCR forward	CAC TCT TTC CCT ACA CGA CGC TCT TCC GAT C

Alpha Chain Reverse 3 + indexing adapters	TGG AGT TCA GAC GTG TGC TCT TCC GAT CT GGGTCAGGGTTCTGGATAT
Beta Chain Reverse 3 + indexing adapters	TGG AGT TCA GAC GTG TGC TCT TCC GAT CT ACACSTTKTTCAGGTCCTC

RT:

42	1hr 30'	
50	2'	2x
42	2'	
70	15'	

Amplification PCR step:

98	3'	
98	20''	26x
67	15''	
72	6'	
72	5'	

First nested PCR (well indexing):

98	4'	
98	20''	21x
67	20''	
72	60''	
72	4'	

Second nested PCR (α/β amplification):

98	4'	
98	20''	10x
67	20''	

72	60''	
72	4'	

Third PCR (plate indexing):

98	4'	
98	20''	6x
65	20''	
72	60''	
72	4'	

9.3. Taqman™ Probes used

All Taqman™ probes were purchased from Life technologies.

gene	code
GAPDH	Hs02758991_g1
SSX2	Hs00817683_m1
NY-ESO	Hs00265824_m1
PRAME	Hs01022301_m1
LAMA3	Hs00165042_m1
FAM122a	Hs00540770_s1
WDR91	Hs00608466_m1
DNAH3	Hs00954323_m1
MYRF	Hs00973739_m1
DNAH9	Hs01017795_m1

EPHA3	Hs00739092_m1
CARD14	Hs01106900_m1
PTPN5	Hs00377920_m1
SLC6A12	Hs00758246_m1
OTOF	Hs00191271_m1
OR2D2	Hs00999189_s1
MASP1	Hs00373559_m1
MCM7	Hs00428518_m1

9.4. Predicted HLA-A2 binding neoantigen peptides bought

Patient	Peptide	gene	mutation	Designation
HGG2	FLEEIILKSL	BDP1	chr5.71553194.G>T	H2A1
HGG2	FLRESQNPL	GRIN3A	chr9.101737294.G>A	H2A2
HGG2	GLALGTPLSI	AMER3	chr2.130763424.C>T	H2A3
HGG2	GLAVNLSQI	KIAA1211	chr4.56315319.C>T	H2A4
HGG2	GNLPDIEVRL	RP5	chr17.40656487.T>C	H2A5
HGG2	LLQDGRTLI	LSM1	chr8.38172012.C>T	H2A6
HGG2	KEMTDITIKA	ADGRV1	chr5.90674196.C>T	H2A7
HGG2	TLCVADFGL	TYRO3	chr15.41573327.G>T	H2A8
HGG2	RLWPSSWVS	ADRA1D	NA	H2A9
HGG2	VLAADIQQC	FSIP2	chr2.185738977.C>T	H2A10
HGG2	FLRESQNPLHL	GRIN3A	chr9.101737294.G>A	H2A11
HGG2	AVEDELLQAV	KBTBD13	chr15.65077036.C>T	H2A12
HGG2	TMLISILKL	C6orf120	chr6.169702993.A>G	H2B1
HGG2	YILVDIDDTF	RP11	chr10.73807430.A>G	H2B2
HGG2	KTLSHLQTL	CD180	chr5.67183662.T>G	H2B3
HGG2	RLWPSSWV	ADRA1D	NA	H2B4
HGG2	NLAEAQSAA	GRB10	chr7.50605336.G>A	H2B5
HGG2	FVMRNHDVV	C9	chr5.39311316.C>T	H2B6
HGG2	HQFNLAETL	MIER2	chr19.334497.A>G	H2B7
HGG2	QVFDFKLSDV	AKR1B15	chr7.134577729.A>T	H2B8
HGG2	LLALMTVAM	PTGIR	chr19.46623486.A>G	H2B9
HGG2	NLAEAQSAAL	GRB10	chr7.50605336.G>A	H2B10
HGG2	LVSDVSVDSV	VWF	chr12.6016143.T>A	H2B11
HGG2	MLAEDMTLCVA	TYRO3	chr15.41573327.G>T	H2B12
HGG2	KLDYLEEKA	CMYA5	chr5.79737699.A>T	H2C1
HGG2	VLLQDGRTL	LSM1	chr8.38172012.C>T	H2C2

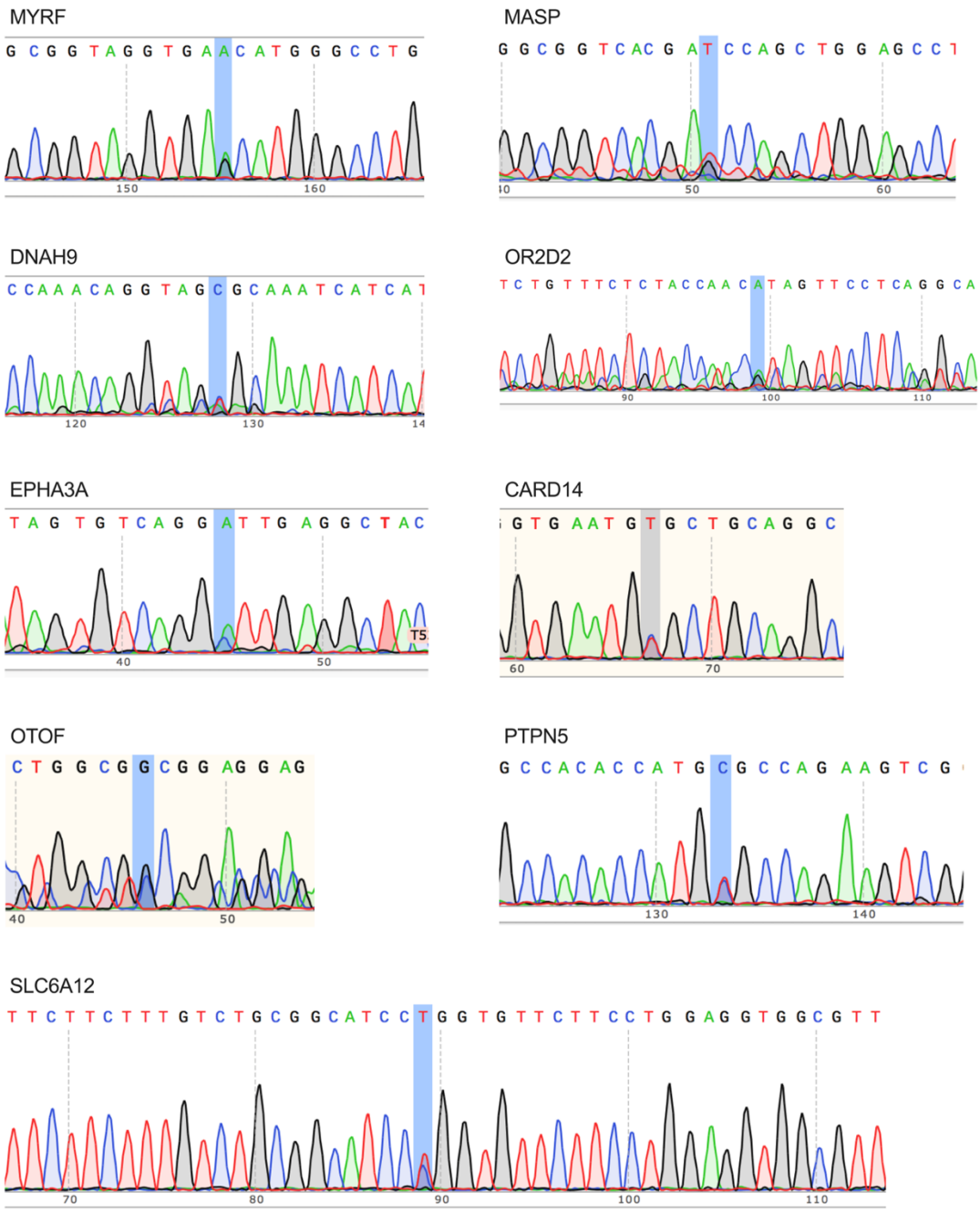
HGG2	AALAGGLYEYV	AQP4	chr18.26856458.A>G	H2C3
HGG2	YITAAYVEV	MCM7	chr7.100094259.T>C	H2C4
HGG2	LLLERGYDVNL	TANC1	chr2.159219266.G>A	H2C5
HGG2	EMTDITIKA	ADGRV1	chr5.90674196.C>T	H2C6
HGG2	ALAGGLYEYVF	AQP4	chr18.26856458.A>G	H2C7
HGG2	ALAGGLYEY	AQP4	chr18.26856458.A>G	H2C8
HGG2	ALFCHQYDI	FNDC5	chr1.32864776.C>T	H2C9
HGG2	ILVSDVSVDSV	VWF	chr12.6016143.T>A	H2C10
HGG2	AVAQDYAQGV	SIPA1L3	chr19.38119638.T>C	H2C11
HGG2	RLLLERGYDV	TANC1	chr2.159219266.G>A	H2C12
HGG2	FKLSDVEMAT	AKR1B15	chr7.134577729.A>T	H2D1
HGG2	ILVSDVSV	VWF	chr12.6016143.T>A	H2D2
HGG2	ALQQLTTHM	TSHZ2	chr20.53254629.A>G	H2D3
HGG2	ILFQCMPVPV	WDR91	chr7.135208809.C>T	H2D4
HGG2	VVVYGMEYL	ECE2	chr3.184285061.T>A	H2D5
HGG2	GRVIENLAEA	GRB10	chr7.50605336.G>A	H2D6
HGG2	LLLERGYDV	TANC1	chr2.159219266.G>A	H2D7
HGG2	SVATGDVIIFL	GALNT10	chr5.154376409.C>T	H2D8
HGG2	ILFSAAKHSV	COMMD4	chr15.75339033.C>T	H2D9
HGG2	FVMRNHDVVL	C9	chr5.39311316.C>T	H2D10
HGG2	ILNKTSGPMEV	POLR1E	chr9.37489350.A>C	H2D11
HGG2	IIPPMFGTV	ACER3	chr11.76926604.G>A	H2D12
HGG2	ILVDIDDTF	RP11	chr10.73807430.A>G	H2E1
HGG2	YLLTGQQKT	DLX6	chr7.97007638.A>G	H2E2
HGG2	KLSDVEMAT	AKR1B15	chr7.134577729.A>T	H2E3
HGG7	AAIESFVSV	ALOXE3	chr17.8108578.T>A	H7A1
HGG7	LMAPLSPGA	SRRT	NA	H7A2
HGG7	LEMDDFEIVV	KCNJ18	chr17.21703655.C>T	H7A3
HGG7	GMLKTDEGI	PRDX2	chr19.12800208.C>T	H7A4
HGG7	EMDDFEIVV	KCNJ18	chr17.21703655.C>T	H7A5
HGG7	LLMRYLKAI	LTN1	NA	H7A6
HGG7	LLLHSSNLI	CCDC150	NA	H7A7
HGG7	AMGNELIQVL	RFX8	chr2.101402438.T>C	H7A8
HGG7	GLSSVALAFL	ABCA8	chr17.68929562.A>G	H7A9
HGG7	KMVRLDLLM	LTN1	NA	H7A10
HGG7	ALTGLVFL	SLC6A18	chr5.1244223.G>T	H7A11
HGG7	MMVNFVVEC	OR5R1	chr11.56417968.G>A	H7A12
HGG7	TAMGNELIQV	RFX8	chr2.101402438.T>C	H7B1
HGG7	QLMAPLSPGA	SRRT	NA	H7B2

HGG7	ALSTVRVGA	CLEC14A	chr14.38255826.G>A	H7B3
HGG7	ILWFEAGEISL	OR51F1	chr11.4769644.G>C	H7B4
HGG7	SQIFFSLHL	OR7C1	NA	H7B5
HGG7	TLTSWSLAA	ATAD3B	chr1.1495668.A>G	H7B6
HGG7	SLFLLYGLSSV	ABCA8	chr17.68929562.A>G	H7B7
HGG7	RLDLLMRYL	LTN1	NA	H7B8
HGG7	SLHLDAWTI	OR7C1	NA	H7B9
HGG7	RLLLHSSNL	CCDC150	NA	H7B10
HGG7	TIWVDLLRA	PARG	chr10.49932102.G>A	H7B11
HGG7	HLDAWTIYS	OR7C1	NA	H7B12
HGG7	FLLYGLSSVAL	ABCA8	chr17.68929562.A>G	H7C1
HGG7	LKLLAEELNQL	PSTK	chr10.122990224.A>C	H7C2
HGG7	GLSSVALAF	ABCA8	chr17.68929562.A>G	H7C3
HGG7	KIEGGIHTA	RTCA	chr1.100268258.A>G	H7C4
HGG7	LFLLYGLSSV	ABCA8	chr17.68929562.A>G	H7C5
HGG7	LLYGLSSV	ABCA8	chr17.68929562.A>G	H7C6
HGG7	LLAEELNQLKA	PSTK	chr10.122990224.A>C	H7C7
HGG7	VLLAPPATGM	PLXNA2	chr1.208217826.C>T	H7C8
HGG7	TIMNTQLWV	OR4D2	chr17.58170058.G>A	H7C9
HGG7	RLSEDYGML	PRDX2	chr19.12800208.C>T	H7C10
HGG11	WLSEDEIML	PTEN	NA	H11A1
HGG11	YLSAIMPSQL	FIGN	chr2.163609698.C>A	H11A2
HGG11	LLAPFSEEGL	E4F1	chr16.2223750.C>T	H11A3
HGG11	VLLAMAFDHYV	OR51E1	chr11.4652900.G>A	H11A4
HGG11	LAMAFDHYV	OR51E1	chr11.4652900.G>A	H11A5
HGG11	QWLSEDEIML	PTEN	NA	H11A6
HGG11	ALAPSGFLGLL	E4F1	chr16.2223750.C>T	H11A7
HGG11	YLDEPFWAQ	SCN10A	chr3.38750124.G>A	H11A8
HGG11	ALKSDFKLV	MAML3	NA	H11A9
HGG11	ILPDFLPI	PAK5	NA	H11A10
HGG11	VLLYCIAAL	SYPL1	chr7.106097768.G>T	H11A11
HGG11	FLEAFHLV	PPEF2	chr4.75860764.C>T	H11A12
HGG11	YIRFFITYV	FADS2	chr11.61863020.A>G	H11B1
HGG11	IIFFFLNRV	ZNF880	NA	H11B2
HGG11	VLSSVNSNLL	NOX1	chrX.100843369.G>T	H11B3
HGG11	KVDHKGPQI	AHNAK2	chr14.104945729.C>T	H11B4
HGG11	DIVGFFFSV	GK5	NA	H11B5
HGG11	MLDSRSTNRV	KLHDC2	chr14.49777866.G>A	H11B6
HGG11	ILPDFLPII	PAK5	NA	H11B7

HGG11	MLQQFTVKL	PTEN	NA	H11B8
HGG11	FLPIITHI	PAK5	NA	H11B9
HGG11	KLVSLRTSL	MAML3	NA	H11B10
HGG11	LVELDETSQEL	DLGAP5	chr14.55188974.C>T	H11B11
HGG11	LLAMAFDHYV	OR51E1	chr11.4652900.G>A	H11B12
HGG11	IMLQQFTVKL	PTEN	NA	H11C1
HGG11	SLFFFFGV	CHL1	NA	H11C2
HGG11	GLLAPFSEEGL	E4F1	chr16.2223750.C>T	H11C3
HGG11	KLPDLWCKI	LAMA3	chr18.23909188.C>T	H11C4
HGG11	FLNILYPKT	SLITRK4	chrX.143630878.A>C	H11C5
HGG11	ALQEEQNIL	CCDC7	chr10.32456320.A>C	H11C6
HGG11	FLLICGLQQV	GLT1D1	chr12.128926413.G>A	H11C7
HGG11	ELDETSQEL	DLGAP5	chr14.55188974.C>T	H11C8
HGG11	REMIPAVLPL	AP4E1	chr15.50925164.T>C	H11C9
HGG11	LLLGDMDQGI	WBSCR22	chr7.73687050.G>A	H11C10
HGG13	YFCLSPPPTLL	DNAH2	NA	H13A1
HGG13	IMEEVWYFL	DNAH3	chr16.20963718.G>A	H13A2
HGG13	IVVEFSSPV	RARS2	NA	H13A3
HGG13	LLPAHYETL	CHN2	chr7.29509358.C>T	H13A4
HGG13	CLSPPPTLL	DNAH2	NA	H13A5
HGG13	KVEEALVLL	EME2	chr16.1775397.G>A	H13A6
HGG13	FLGITNML	FAM122A	chr9.68780820.A>T	H13A7
HGG13	RLQPDRVAM	ACO2	chr22.41507892.G>A	H13A8
HGG13	GMVLAEDGV	ALK	chr2.29226954.G>A	H13A9
HGG13	TVMTITVYA	CEACAM5	chr19.41715887.C>T	H13A10
HGG13	IQLAVFFFL	CD34	NA	H13A11
HGG13	GLRVVAIQV	FGF13	chrX.138703039.C>A	H13A12
HGG13	LLLPAHYETL	CHN2	chr7.29509358.C>T	H13B1
HGG13	FCLSPPPTLL	DNAH2	NA	H13B2
HGG13	SQIFFSLHL	OR7C1	NA	H13B3
HGG13	GTIGLFLPL	CHRNA2	chr8.27461651.G>A	H13B4
HGG13	HLEQEEAFL	TRIO	chr5.14369506.A>G	H13B5
HGG13	SLHLDAWTI	OR7C1	NA	H13B6
HGG13	HLDAWTIYS	OR7C1	NA	H13B7
HGG13	LLLPAHYET	CHN2	chr7.29509358.C>T	H13B8
HGG13	VLHFPLVNC	PLA2G4F	chr15.42142555.G>A	H13B9
HGG13	FFLGITNML	FAM122A	chr9.68780820.A>T	H13B10
HGG13	QLAVFFFLL	CD34	NA	H13B11
HGG13	LLGFSHLANL	OR10C1	chr6.29440076.G>A	H13B12

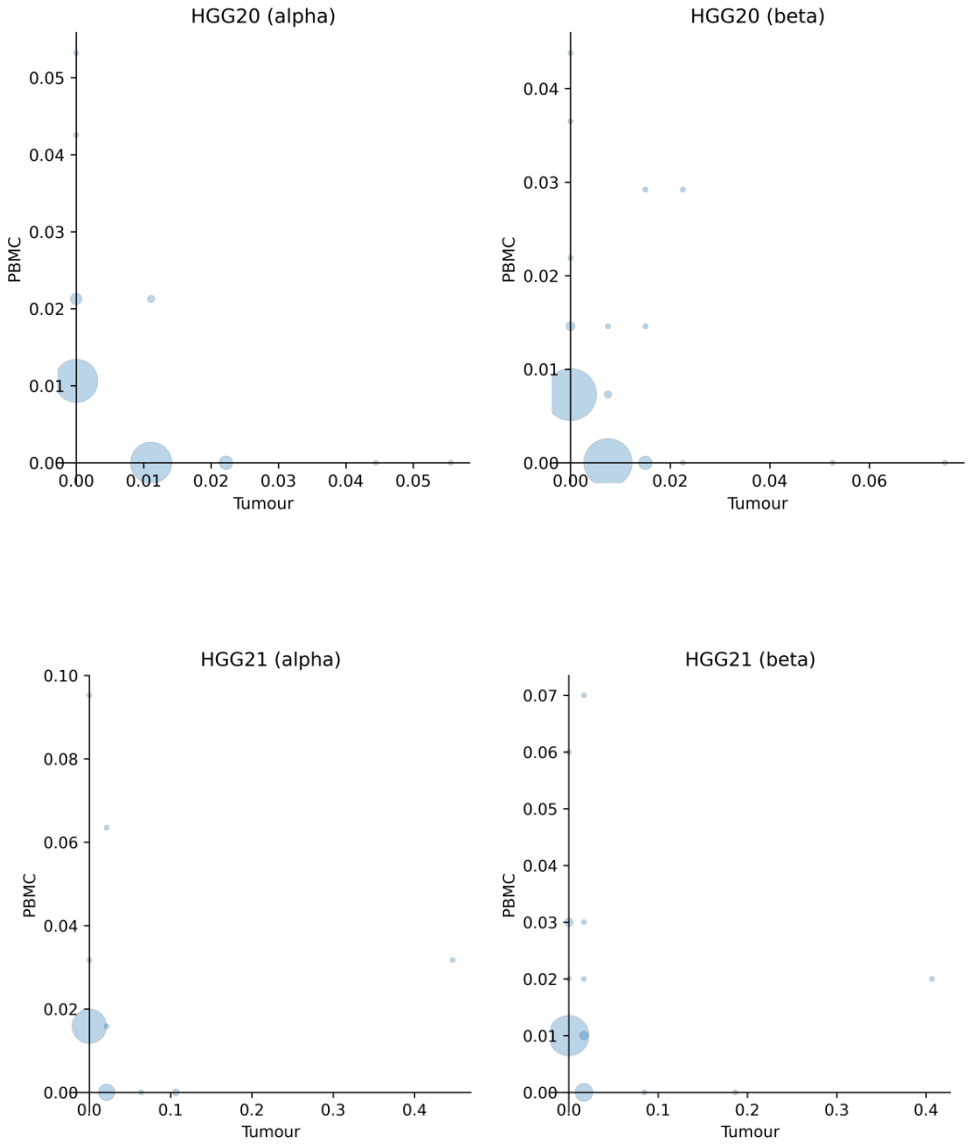
HGG13	FLTGISQNYA	DNAH3	chr16.20935410.C>T	H13C1
HGG13	ALGALLILQL	NOTCH4	chr6.32199062.C>T	H13C2
HGG13	IVFPIIVIVV	SLC6A16	chr19.49293272.C>T	H13C3
HGG13	YLRLQPDRV	ACO2	chr22.41507892.G>A	H13C4
HGG13	YLDATFSPCL	PITRM1	chr10.3165440.A>G	H13C5
HGG13	NIYPNIIAM	PTEN	chr10.87864548.T>A	H13C6
HGG13	AVVGPLPTM	NOTCH2	chr1.119915724.G>A	H13C7
HGG13	TLAELVRYYM	PTPN11	chr12.112450416.A>G	H13C8
HGG13	VMFIGVNLT	MT-COX1	chrM.7149.A>G	H13C9
HGG13	TVMFIGVNL	MT-COX1	chrM.7149.A>G	H13C10
U87	SLADLCFSTNV	OR2D2	Chr11.6913505.T>C	U87 1
U87	SLADLCFSTNVV	OR2D2	Chr11.6913505.T>C	U87 2
U87	TVADFWHMV	PTPN5	Chr11.18754846.C>T	U87 3
U87	GILVFFLEV	SLC6A12	Chr12.313816.G>A	U87 4
U87	ILVFFLEVAL	SLC6A12	Chr12.313816.G>A	U87 5
U87	KELPMFTYRV	MYRF	Chr11.61541446.C>T	U87 6
U87	STVADFWHMV	PTPN5	Chr11.18754846.C>T	U87 7
HGG2	YITAAYVEM	MCM7	chr7.100094259.T>C	H2C4 WT
HGG2	VLFQCMPVPV	WDR91	chr7.135208809.C>T	H2D4 WT
HGG11	KLPDLWRK	LAMA3	chr18.23909188.C>T	H11C4 WT
HGG13	ITEEVWYFL	DNAH3	chr16.20963718.G>A	H13A2 WT
HGG13	FQGITNML	FAM122A	chr9.68780820.A>T	H13A7 WT

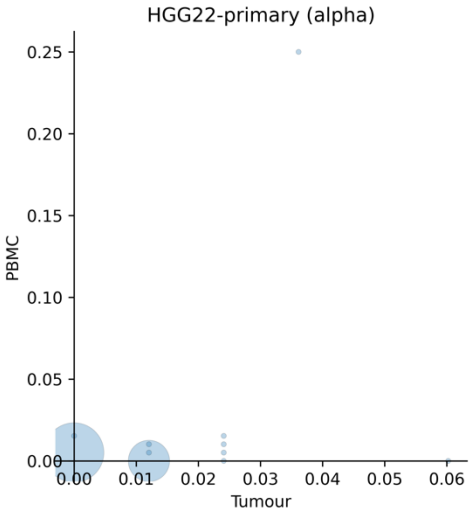
9.5. Sanger sequencing of U87MG cell line

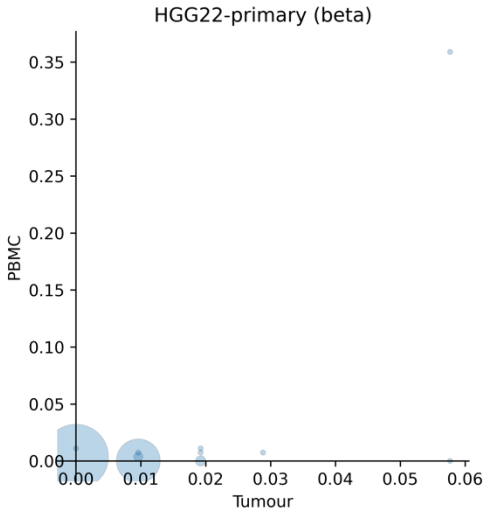


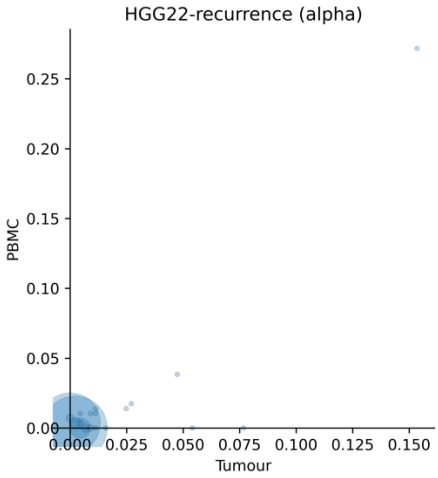
Predicted mutations are present in U87MG cell line. Sanger sequencing confirms the presence of mutations described in CCLE predicted to form potential neoantigens in U87MG cell line.

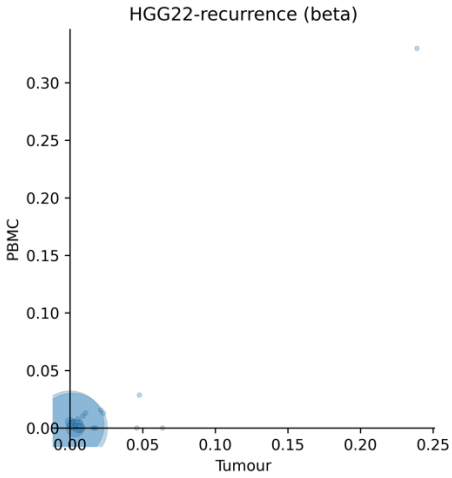
9.6. TIL/PBMC TCR repertoire overlap of individual patient samples

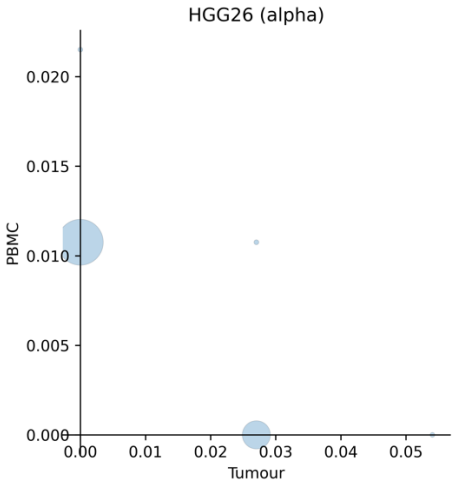


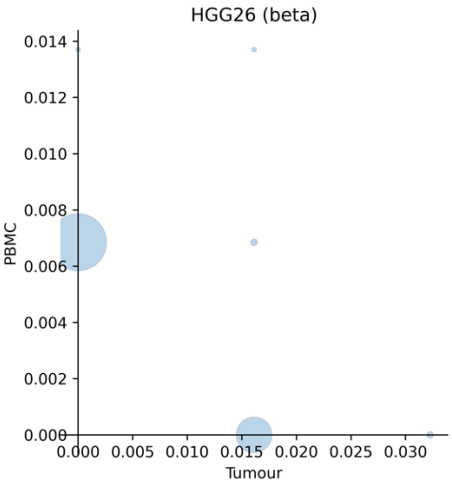

HGG22-primary (alpha)
PBMC
Tumour
0.25
0.20
0.15
0.10
0.05
0.00
0.00
0.01
0.02
0.03
0.04
0.05
0.06

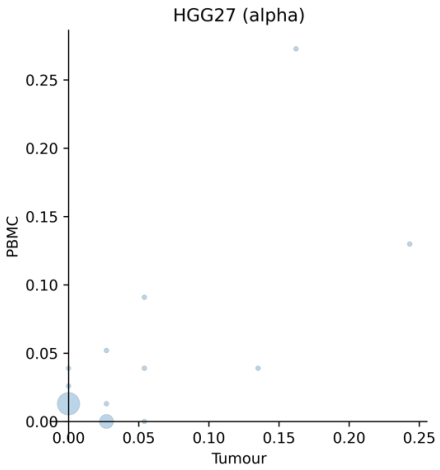

HGG22-primary (beta)
PBMC
Tumour
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.00
0.01
0.02
0.03
0.04
0.05
0.06

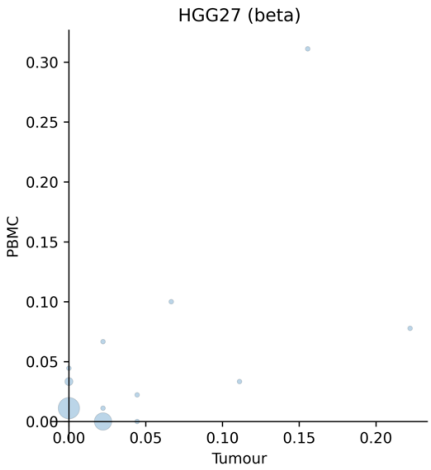

HGG22-recurrence (alpha)
PBMC
Tumour
0.25
0.20
0.15
0.10
0.05
0.00
0.000
0.025
0.050
0.075
0.100
0.125
0.150

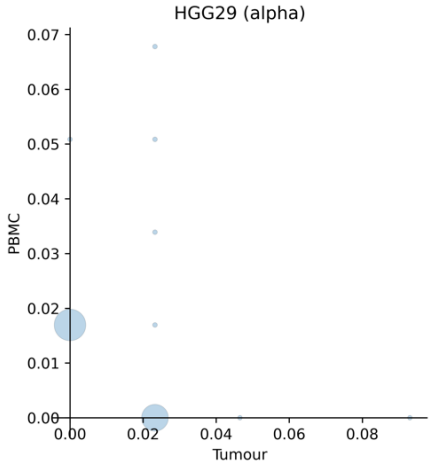

HGG22-recurrence (beta)
PBMC
Tumour
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.00
0.05
0.10
0.15
0.20
0.25

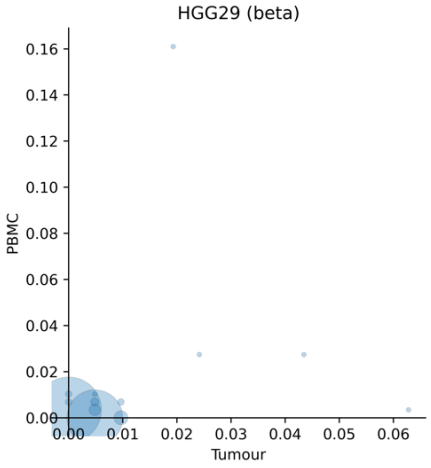

HGG26 (alpha)
PBMC
Tumour
0.020
0.015
0.010
0.005
0.000
0.00
0.01
0.02
0.03
0.04
0.05

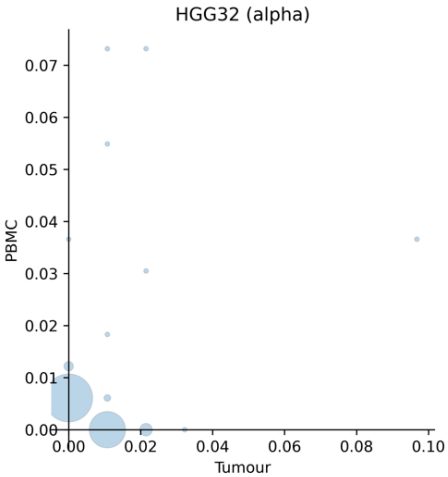

HGG26 (beta)
PBMC
Tumour
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
0.000
0.005
0.010
0.015
0.020
0.025
0.030

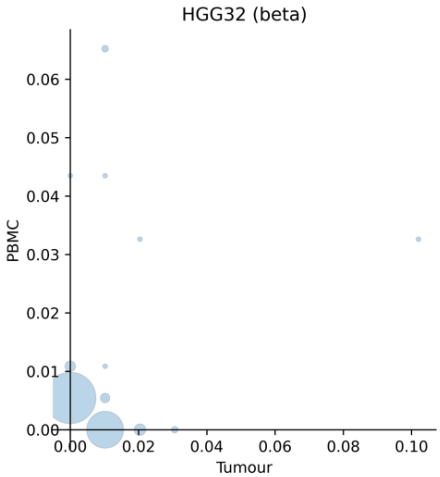

HGG27 (alpha)
PBMC
Tumour
0.00
0.05
0.10
0.15
0.20
0.25

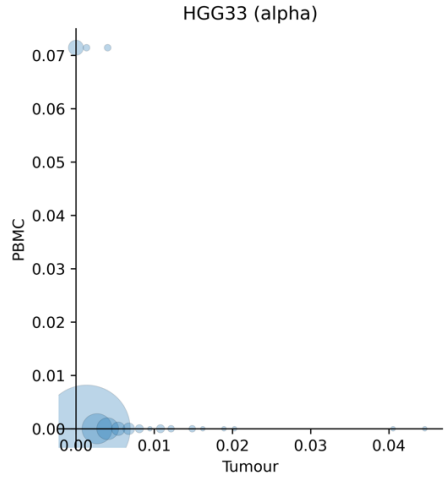

HGG27 (beta)
PBMC
Tumour
0.00
0.05
0.10
0.15
0.20
0.25
0.30

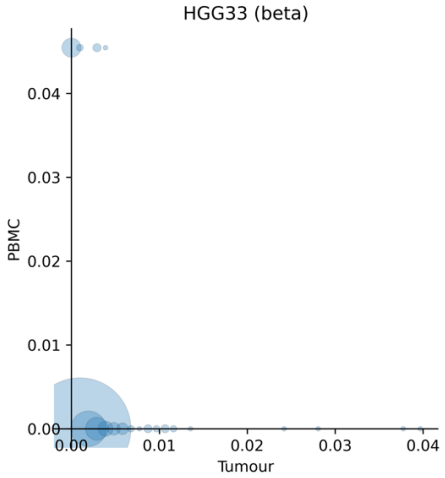

HGG29 (alpha)
PBMC
Tumour
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08

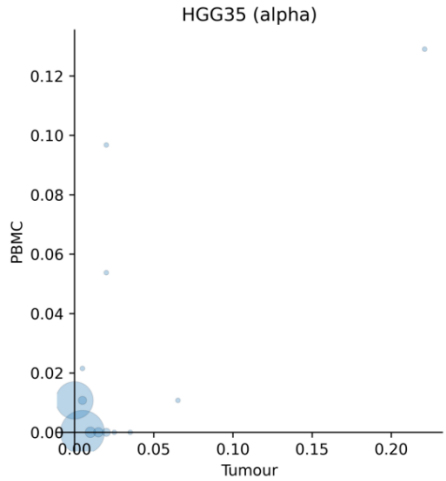

HGG29 (beta)
PBMC
Tumour
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16

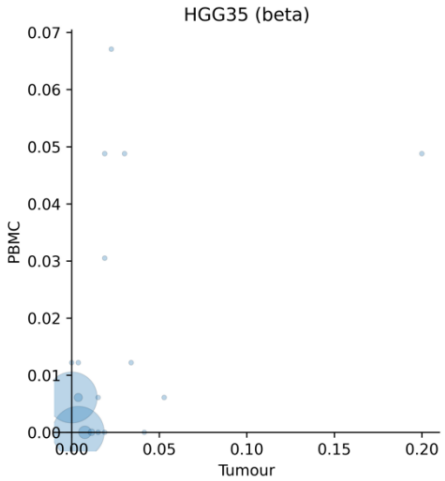

HGG32 (alpha)
PBMC
Tumour
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.10

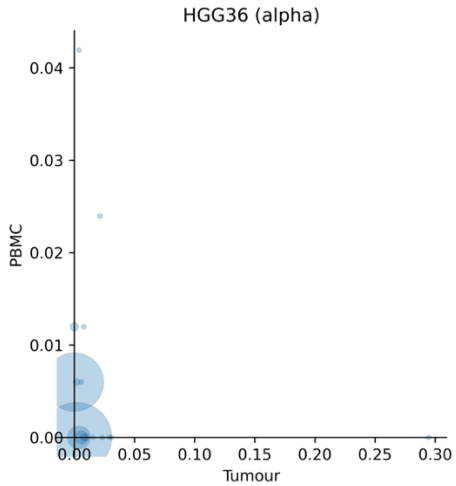

HGG32 (beta)
PBMC
Tumour
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.08
0.10

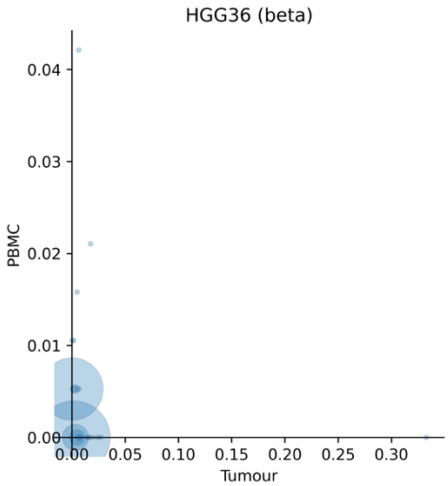

HGG33 (alpha)
PBMC
Tumour
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07

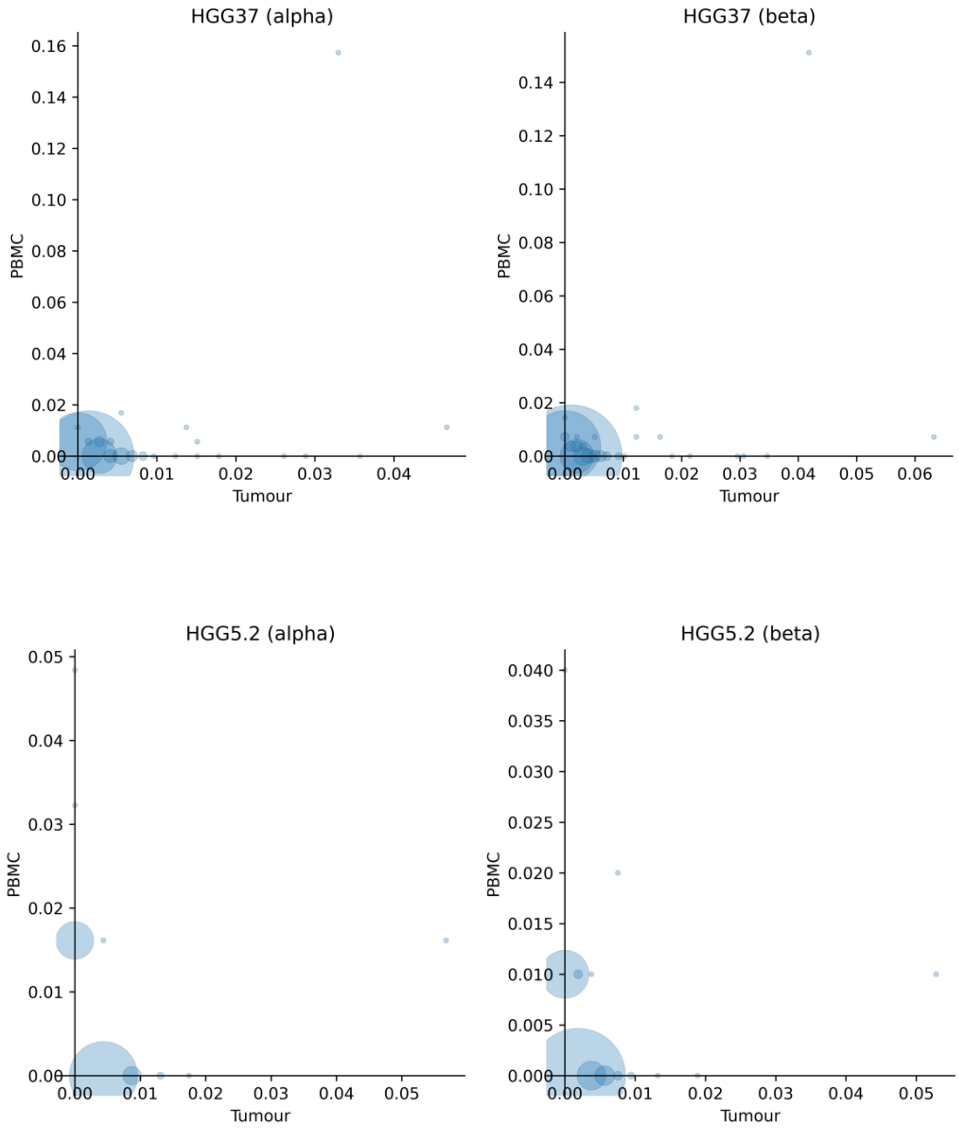

HGG33 (beta)
PBMC
Tumour
0.00
0.01
0.02
0.03
0.04


HGG35 (alpha)
PBMC
Tumour
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.05
0.15
0.20


HGG35 (beta)
PBMC
Tumour
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.10
0.15
0.20

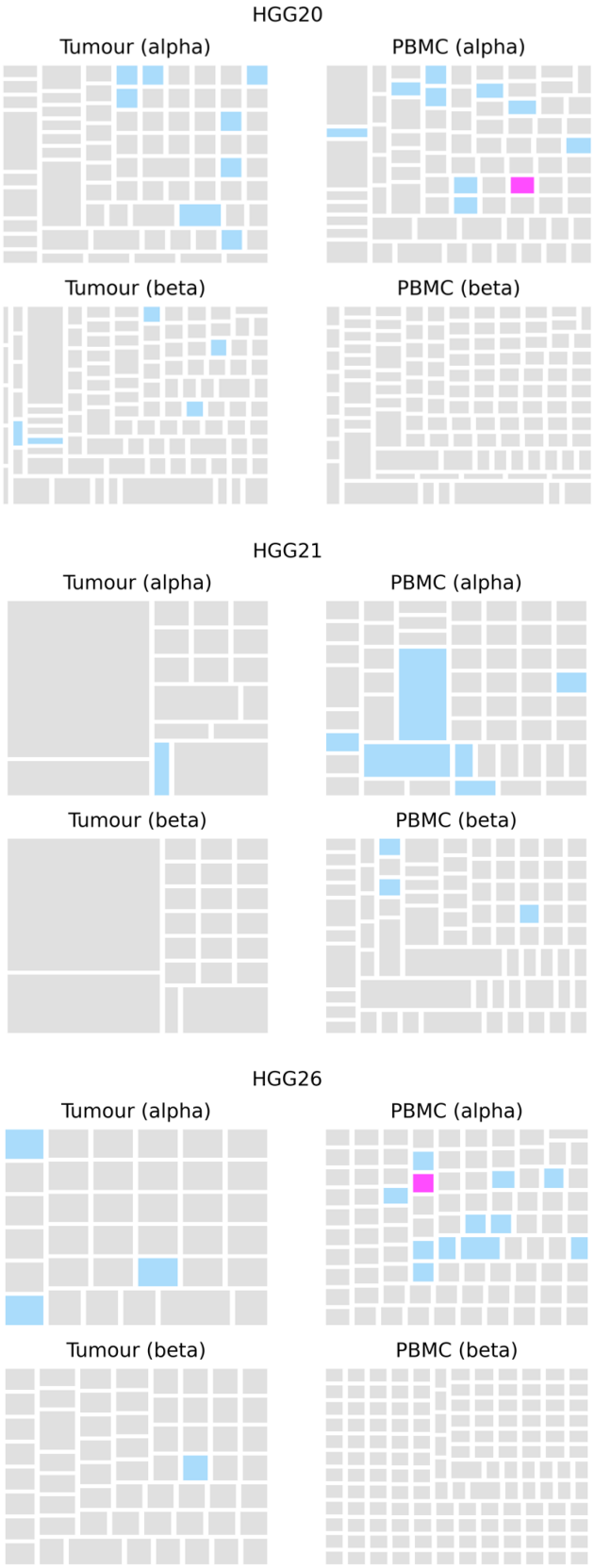

HGG36 (alpha)
PBMC
Tumour
0.00
0.01
0.02
0.03
0.04
0.05
0.10
0.15
0.20
0.25
0.30


HGG36 (beta)
PBMC
Tumour
0.00
0.01
0.02
0.03
0.04
0.05
0.10
0.15
0.20
0.25
0.30



Dot plots showing overlap of TCR between PBMC and TIL samples of individual patients. X and Y axis represent proportion of repertoire TCR makes up. Size of dot represents number of unique TCR for that position.

9.7. Analysis of public TCR in individual patient samples



HGG27

Tumour (alpha)

PBMC (alpha)

Tumour (beta)

PBMC (beta)

HGG27.2

Tumour (alpha)

PBMC (alpha)

Tumour (beta)

PBMC (beta)

HGG29

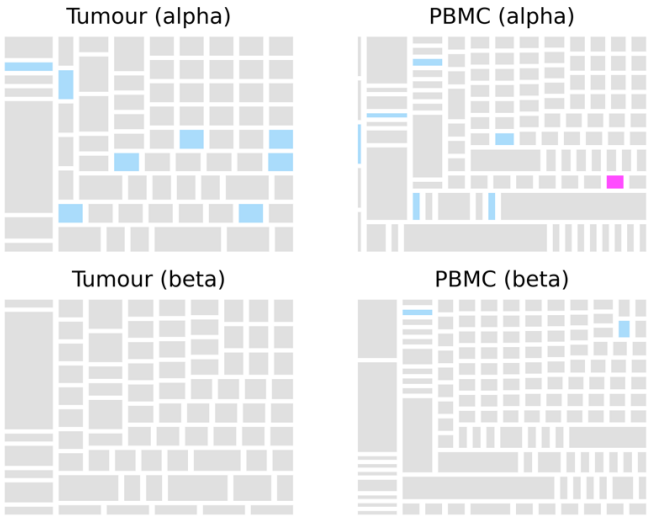
Tumour (alpha)

PBMC (alpha)

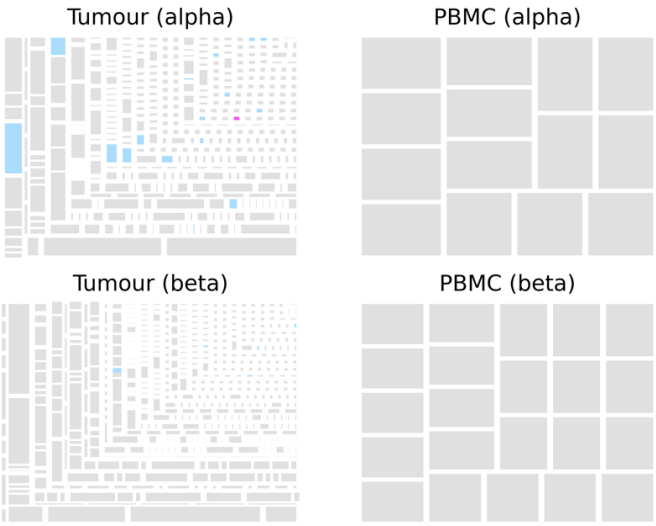
Tumour (beta)

PBMC (beta)

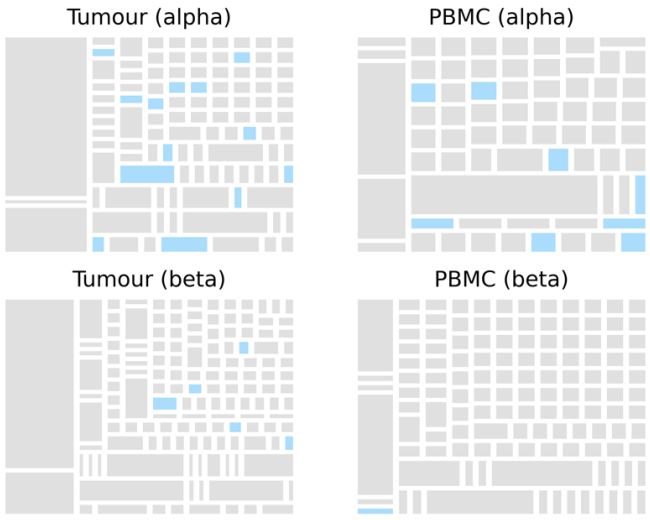
HGG32

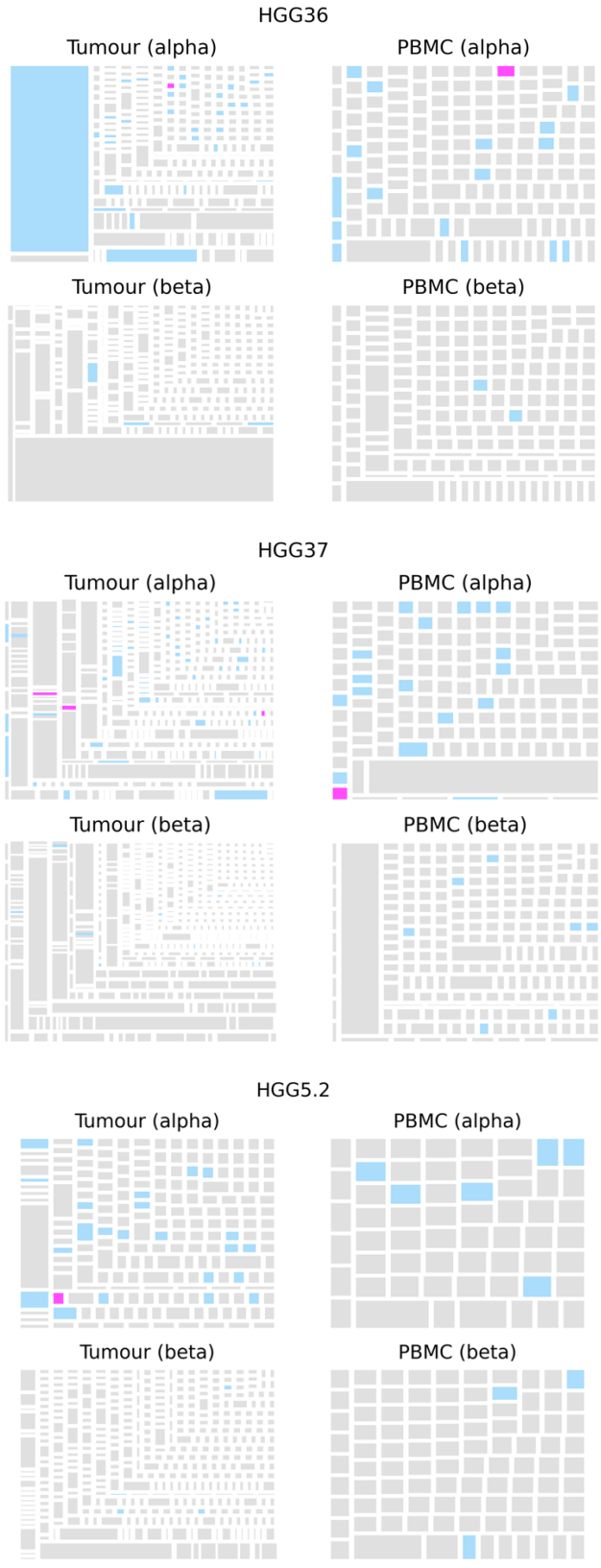


HGG33



HGG35





Identification of public TCR in repertoire of PBMC/TIL of individual patient samples. Box plots showing TCR repertoire and public TCR as found on VDJdb. Each box represents a unique TCR. Box size proportional to size of TCR clone. Blue – viral specific TCR, magenta – cancer associated antigen specific TCR.