The Sir Peter MacCallum Department of Oncology
CONFIRMATION REPORT

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Abstract

Chimeric antigen receptor (CAR) T cell therapy has been highly efficacious in the treatment of haematological malignancies; however, these successes have not been reciprocated in solid cancers. CAR T cells face various barriers in solid cancers that limit their therapeutic efficacy, including tumour heterogeneity, inefficient trafficking into the tumour, and immunosuppressive mechanisms in the tumour microenvironment. Engineering CAR T cells to express immunomodulatory factors, such as pro-inflammatory cytokines, is a strategy that has the potential to simultaneously overcome multiple limitations of CAR T cell therapy through eliciting widespread effects on endogenous immune mechanisms. While these so-called "armoured" CAR T cells have demonstrated promising results in preclinical models, their clinical application been hampered by severe toxicities due to unregulated transgene expression. CRISPR/Cas9 gene editing offers the potential to improve the design and efficacy of armoured CAR T cells through the ability to integrate transgenes into specific genomic sites, hence enabling transgene regulation through an endogenous tumour-specific promoter. As T cells often upregulate inhibitory genes such as PD-1 in response to antigen recognition in the tumour, insertion of the transgene into such loci would confer a two-fold benefit of disrupting a suppressive gene while obtaining tumour-specific expression of the pro-inflammatory transgene. This project involves the development of a novel strategy to utilise CRISPR/Cas9 gene editing to fine-tune genetic circuitry in armoured CAR T cells.

Section 1. The Immune System and Cancer

The immune system is composed of a complex interplay of cells, molecules and tissues that orchestrate a coordinated response against foreign particles. This is broadly classified into two main arms, innate and adaptive immunity, which execute distinct functions but are intrinsically linked to facilitate a strong defence against pathogens and malignant cells. The mid-twentieth century saw the initial discoveries which demonstrated the protective role of the immune system in cancer (Dunn et al., 2002), paving the way for the development of cancer immunotherapy, a new therapeutic modality in cancer treatment.

1.1 – The innate immune system

The innate immune system is the first line of defence to pathogens and foreign molecules, and provides an acute, non-specific response to both internal and external threats. This is dependent on the recognition of common molecular patterns associated with pathogens and cellular abnormalities. Upon detection, a rapid immune response is mounted, involving the secretion of cytokines, which are signalling molecules, and chemokines, a subset of chemotactic cytokines responsible for the recruitment of immune cells, to coordinate an appropriate response to the threat. Another key role of the innate immune system is to initiate an adaptive immune response through the function of dendritic cells (DCs), a type of professional antigen presenting cell (APC). DCs possess the ability to internalise and process antigens for the activation of T lymphocytes, which are responsible for coordinating a robust and specific response (discussed in Section 1.2.1).

1.2 – The adaptive immune system

While the innate immune response is rapid and short-lived, the adaptive immune response is slower to develop, but eliminates foreign threats with high specificity and provides long-term protection through the development of memory. The adaptive immune system, broadly classified into antibody-dependent humoral immunity, mediated by B lymphocytes, and T lymphocyte-mediated cellular immunity, is dependent on the recognition of specific antigens by cell surface receptors. A diverse repertoire of antigen-specific receptors is developed through a process of gene rearrangement and stochastic DNA modifications in the receptor-encoding genomic region in both B and T lymphocytes during their development. In the presence of a foreign antigen, the generation of an antigen-specific response occurs through the selection, amplification and differentiation of B and T cell clones that express a high-affinity receptor to the specific antigen. This review will focus on T lymphocytes.

1.2.1 – T lymphocytes

T lymphocytes eliminate cells expressing foreign antigens, including pathogen-infected cells and transformed cells expressing mutated proteins. Each T cell expresses a T cell receptor (TCR) that is specific to a single antigen. In their naïve state, T cells circulate through secondary lymphoid organs, which consist of lymph nodes and the spleen, and undergo apoptosis in the absence of antigenic stimulation after a few days. However, upon TCR binding to its cognate antigen presented on APCs, T cells differentiate into effector and memory cells and home to sites of inflammation to mediate cell killing.

CD8+ T cells

$CD8^+$ T cells mediate the direct killing of antigen-expressing cells through the release of cytotoxic granules or expression of death ligands (Martínez-Lostao et al., 2015). In the former process, pre-formed cytoplasmic granules containing perforin, a pore-forming protein, and granzymes, which are serine proteases, are exocytosed towards the target cell following TCR binding to antigen (De Saint Basile et al., 2010). Perforin assists in the delivery of granzymes to the target cell through the formation of pores in the cell membrane, and granzymes mediate cell death through the cleavage of intracellular proteins (Voskoboinik et al., 2015). While apoptosis is commonly considered to be the main mechanism of granzyme-mediated cell death, only granzyme B has been shown to induce apoptosis (Pardo et al., 2009), and other granzymes may facilitate cell death through other mechanisms (Martínez-Lostao et al., 2015). The other mechanism of $CD8^+$ T cell-mediated cell death is the expression of death ligands, either on the T cell membrane or secreted in exosomes. Binding of death ligands such as Fas ligand or TRAIL (TNF-related apoptosis inducing ligand) leads to caspase-dependent apoptotic cell death (Walczak, 2013).

CD4+ T cells

CD4+ T cells differentiate into T helper (Th) cells following activation, and coordinate cellular immunity through providing help to various cell subsets, including CD8+ T cells. Of the various Th cell subsets, Th1 cells are understood to be the predominant subset in mediating anti-tumour immunity (Ostroumov et al., 2018), as they produce interferon-γ (IFN-γ) to enhance the cytotoxicity of CD8+ T cells, and to broadly promote inflammation. CD4+ T cells assist in CD8+ T cell differentiation through enhancing the ability of DCs to activate CD8+ T cells (Ridge et al., 1998), and through the production of IL-2 (Wilson and Livingstone, 2008), leading to improved clonal expansion of CD8+ T cells, cytotoxic potential and differentiation into effector and memory CD8+ T cells (Borst et al., 2018). While CD8+ T cells are generally regarded to be the key T cell subset that

contributes to anti-tumour immunity, there is evidence that CD4+ T cells are also required for optimal anti-tumour immunity (Audun et al., 2014).

Antigen Presentation to T Cells

T cells recognise their cognate antigens when presented by APCs or target cells on major histocompatibility complex (MHC) molecules. There are two canonical classes of MHC molecules, MHC class I, which presents antigens to CD8+ T cells, and MHC class II, which presents antigens to CD4+ T cells. MHC I molecules are expressed on all nucleated cells, and present intracellular antigens, such as viral peptides or mutated peptides in a malignant cell. This is the key mechanism by which cells are recognised by CD8+ T cells and subsequently subjected to CD8+ T cell-mediated killing. MHC II molecules are expressed on APCs, including DCs, macrophages and B cells, and present extracellular antigens, such as peptides from pathogens that have been endocytosed. MHC II presentation serves to activate CD4+ T cells to initiate the adaptive immune response. A specialised class of DCs, known as conventional type 1 DCs (cDC1s), are capable of processing and redirecting extracellular antigens for MHC I presentation, a process known as antigen cross-presentation, enabling the activation of naïve CD8+ T cells. Importantly, cDC1s have recently been demonstrated to play an essential role in cancer immunity and the success of T cell-based immunotherapies, as the key DC subset capable of initiating *de novo* anti-tumour T cell responses (Broz et al., 2014; Roberts et al., 2016; Salmon et al., 2016).

T Cell Activation by DCs

The activation of naïve T cells by DCs requires three signals to ensure the development of robust effector functions. Signal 1 occurs through TCR binding to its cognate antigen presented on an MHC molecule. Signal 2 is obtained through the activation of costimulatory receptors, such as CD28 on T cells binding to its ligands B7.1 and B7.2, expressed on APCs. Signal 3 is provided through APC-derived cytokines, which direct T cell differentiation. For example, the production of IL-12 by DCs upon CD4+ T cell activation leads to the differentiation of Th1 cells.

1.3 – The role of the immune system in cancer

Following evidence that mice can acquire resistance to tumours derived from the same genetic background (Klein et al., 1965; Old and Boyse, 1963), Burnet and Thomas proposed the “cancer immunosurveillance” hypothesis, describing the antigenic nature of tumours and the ability of the immune system to detect and eradicate these cells (Burnet, 1970, 1964, 1957; Thomas, 1982). The concept of “cancer immunoediting” was proposed by Dunn et al. in 2002 to describe the dynamic relationship between tumours and the immune system over the course of oncogenesis. This is summarised into three phases: Elimination, equilibrium and escape (Dunn et al., 2002). During the

elimination phase, detection of neoantigens on emerging tumour cells by the innate immune system initiates an anti-tumour immune response, characterised by local inflammation, antigen uptake by DCs, activation of tumour-specific CD4+ and CD8+ T cells, leading to tumour cell killing. The selective pressure exerted on tumours during this process leads to the development of resistance through the acquisition of genomic mutations. This characterises the equilibrium phase, where emerging resistance to immune-mediated killing prevents complete elimination of the tumour mass. Finally, in the escape phase, tumour cells manage to successfully surpass immune control, and begin to proliferate unchecked. It is often in the escape phase that tumours become clinically detectable.

Section 2. Introduction to Immunotherapy

Based on the understanding that the immune system has an intrinsic ability to detect and eliminate malignant cells, cancer immunotherapy aims to engage or reinvigorate anti-tumour immune mechanisms for the treatment of cancer. Two major forms of immunotherapy which have achieved outstanding clinical success are checkpoint blockade and CAR T cell therapy, which will be discussed in this review.

2.1 - Checkpoint Blockade Therapy

Immune checkpoint blockade therapy utilises monoclonal antibodies targeting inhibitory T cell surface proteins or their cognate ligands expressed on tumour cells. Thus far, monoclonal antibodies targeting two immune checkpoint pathways, the PD-1/PD-L1 axis and the CTLA-4 axis, have been approved by the U.S. Food and Drug Administration (FDA) (Wei et al., 2018). PD-1/PD-L1 checkpoint blockade will be covered in this review.

2.1.1 - PD-1

The PD-1/PD-L1 axis maintains T cell homeostasis by dampening T cell responses following activation, to minimise excessive inflammation. PD-1 is rapidly expressed on T cells upon TCR stimulation (Agata et al., 1996), and has two ligands, PD-L1 and PD-L2. PD-L1 is broadly expressed on normal tissues and commonly expressed on tumour cells, while PD-L2 expression is more restricted, predominantly being expressed on macrophages and DCs (Inaguma et al., 2017; Keir et al., 2006; Parra et al., 2019). Their expression is upregulated in response to inflammatory cytokines, such as IFN-γ, which is abundant in tumours (Chen et al., 2019b). Ligation of PD-1 to PD-L1 or PD-L2 leads to the attenuation of T cell signalling through the recruitment of the tyrosine phosphatase SHP2, which dephosphorylates the TCR downstream signalling proteins (Yokosuka et al., 2012) and the costimulatory molecule CD28 (Hui et al., 2018). Prolonged PD-1 signalling leads to metabolic restriction in T cells, resulting in T cell exhaustion or dysfunction (Patsoukis et al., 2015), a state of reduced cytotoxic capacity (Zhang et al., 2020b). PD-1 and PD-L1 blockade leads to durable

responses across a range of cancers, generally in cancers with high PD-L1 expression such as Hodgkin's lymphoma, and highly immunogenic cancers such as melanoma and Merkel cell carcinoma (Ribas and Wolchok, 2018). Clinical evidence suggests that the efficacy of anti-PD-1 and anti-PD-L1 therapy is dependent on the reactivation of a pre-existing intratumoural T cell response by reversing T cell inhibition via the PD-1/PD-L1 axis (Tumeh et al., 2014).

2.2 – Adoptive cell therapy

Adoptive cell therapy (ACT) is based on the *ex vivo* manipulation and expansion of a patient's T cells for subsequent reinfusion into the patient. While the earliest applications of ACT involved the isolation and expansion of lymphokine activated killer (LAK) cells from peripheral blood or tumour-infiltrating lymphocytes (TILs) from a tumour sample, later forms of ACT, including TCR-modified T cell therapy and CAR T cell therapy, utilised gene manipulation techniques to engineer T cells to express a tumour-specific receptor.

2.2.1. - Chimeric Antigen Receptor (CAR) T Cells

Since their development in the late 1980s, CAR T cell therapy has rapidly become a key therapeutic modality for cancer treatment. A CAR is a synthetic receptor composed of an extracellular antibody-derived single chain variable fragment (scFv) with specificity for the target antigen, fused to intracellular TCR signalling domains. First-generation CARs utilised the TCR signalling domain CD3, responsible for transduction of "Signal 1" following TCR activation. Early studies demonstrated that expression of a CAR in T cells successfully redirected their killing capacity to cells expressing the target antigen, accompanied by IL-2 production (Eshhar et al., 1993; Stancovski et al., 1993). However, the development which dramatically improved their clinical efficacy was the addition of either a CD28 or 4-1BB costimulatory domain to the intracellular region in second-generation CARs, which provided the necessary "Signal 2" for robust T cell activation, leading to improved CAR T cell function (Imai et al., 2004; Maher et al., 2002). Third-generation CARs, which contain two costimulatory domains, are currently being assessed for their ability to further improve clinical efficacy (Enblad et al., 2018; Ramos et al., 2018).

Efficacy of CAR T Cells

Haematological Cancers

There are currently two CAR T cell products that have obtained FDA approval, tisagenlecleucel and axicabtagene ciloleucel, which both target CD19, a B cell surface molecule that is uniformly expressed on the majority of B cell lymphomas and leukaemias at high levels (Uckun et al., 1988). Of note, tisagenlecleucel was approved by the Australian Therapeutic Goods Administration (TGA)

in 2018 for clinical use. The use of CD19-specific CAR T cells in the treatment of B cell acute lymphoblastic leukaemia (B-ALL) have demonstrated impressive results, with complete remission rates as high as 80 – 90%, with significant, albeit lower, remission rates of 30 – 50% in chronic lymphocytic leukaemia (CLL) and non-Hodgkin's lymphoma (NHL) (Grupp et al., 2013; Maude et al., 2018; Park et al., 2018; Porter et al., 2011). As CD19 is expressed on all B cells, on-target off-tumour toxicity leading to B cell aplasia is observed in most patients. However, B cell aplasia is generally manageable through periodic immunoglobulin infusions, and is often considered to be an indication of successful CAR T cell engraftment (Tasian and Gardner, 2015).

Solid Cancers

While anti-CD19 CAR T cells are highly efficacious for the treatment of B cell malignancies, this success has yet to be reciprocated in the context of solid cancers. There are currently over 700 CAR T cell clinical trials registered with the U.S. National Library of Medicine (clinicaltrials.gov), with over 30 target antigens being tested, including Lewis-Y, GD2, EGFR, HER2, and mesothelin. However, successful outcomes remain limited, and examples of the best results include GD2-specific CAR T cells for paediatric neuroblastoma, where complete remission was achieved in 3 out of 11 patients (Louis et al., 2011), and anti-Her2 CAR T cells in the treatment of Her2-positive sarcoma, with 3 out of 17 patients achieving complete remission with surgery following CAR T cell therapy (Ahmed et al., 2015).

Limitations of CAR T Cell Therapy in Solid Tumours

Tumour Heterogeneity

Unlike B cell malignancies such as B-ALL and CLL, which uniformly express CD19 (Uckun et al., 1988), solid tumours are highly heterogeneous, and there is no targetable solid tumour antigen identified so far that exhibits consistent and high expression on tumour cells while also lacking high expression on normal tissues. CAR targets that are currently under investigation range in expression across tumour types, often with expression levels of 20 – 50% (Martinez and Moon, 2019). Hence, CAR T cells targeting a single antigen would be incapable of detecting and eliminating every tumour cell within a tumour mass.

CAR T Cell Trafficking to Tumours

T cells face multiple physical and anatomical barriers that prevent their infiltration of tumours, such as a highly compact stroma and extracellular matrix, and abnormal and disorganised vasculature that precludes a large influx of immune cells into the core of the tumour (Vignali and Kallikourdis, 2017). Furthermore, tumours often express low levels of the chemokines which recruit effector T cells into

the tumour, including CXCL9, CXCL10 and CXCL11, which bind to the receptor CXCR3, and CCL2 and CCL5, which bind to CCR2 and CCR5, respectively (Denkert et al., 2015; Harlin et al., 2009; House et al., 2020; Zumwalt et al., 2014). Specifically, the CXCR3 axis has been demonstrated to be indispensable for CD8+ T cell homing to tumours, as abrogating T cell expression of CXCR3 in an adoptive T cell transfer model has been shown to reduce therapeutic efficacy (Mikucki et al., 2015). Furthermore, our lab has recently demonstrated that blocking CXCR3 leads to significantly reduced T cell infiltration into tumours and reduced tumour control following immune checkpoint blockade therapy (House et al., 2020).

The Immunosuppressive Tumour Microenvironment

The tumour microenvironment (TME) poses another barrier to CAR T cells through multiple immunosuppressive mechanisms. The TME consists of inhibitory immune cells including myeloid-derived suppressor cells (MDSCs), tumour-associated macrophages (TAMs), tumour-associated neutrophils (TANs), and Tregs, which employ various strategies to limit CAR T cell activity (Hanahan and Coussens, 2012; Hinshaw and Shevde, 2019; Motz and Coukos, 2013; Rodriguez-Garcia et al., 2020). These immune cells, as well as tumour cells, produce or facilitate the generation of immunosuppressive factors which have been shown to inhibit CAR T cell function, including adenosine (Beavis et al., 2017; Ohta et al., 2006), indoleamine 2,3-dioxygenase (IDO) (Ninomiya et al., 2015), prostaglandin E2 (PGE2) (Newick et al., 2016), and cytokines such as TGF-β (Kloss et al., 2018; Tang et al., 2020). TGF-β synergises with IL-10, both of which are secreted by Tregs, MDSCs, TAMs and tumour cells, to induce widespread inhibitory effects. This includes the suppression of T cell proliferation, effector functions, differentiation and stabilisation of Tregs and inhibition of antigen presentation through the downregulation of MHC II and costimulatory molecules CD80 and CD86 on APCs (Fu and Jiang, 2018; Motz and Coukos, 2013). The cytokine milieu in the TME also serves to upregulate the expression of checkpoint ligands including PD-L1 (Rodriguez-Garcia et al., 2020). Furthermore, TAMs and MDSCs secrete arginase-1 and IDO, which metabolise arginase and tryptophan, respectively, depleting effector T cells of key metabolites (Gabrilovich and Nagaraj, 2010; Mantovani et al., 2017).

Apart from producing immunosuppressive cytokines, the inhibitory immune cells in the TME also play direct roles in suppressing T cell activity. Tregs directly inhibit T cell function through the expression of CTLA-4 to sequester costimulatory molecules on APCs from effector T cells, and can directly eliminate effector T cells through granzyme and perforin-mediated killing (Togashi et al., 2019). TAMs have been shown to physically associate with CD8+ T cells to prevent their infiltration into the tumour (Peranzoni et al., 2018).

Armoured CAR T Cells: Fourth Generation CARs

Armoured CAR T cells, sometimes considered fourth generation CARs, have the potential to simultaneously address multiple limitations of CAR T cell therapy. This strategy involves the further engineering of CAR T cells to express immunomodulatory factors such as pro-inflammatory cytokines or costimulatory ligands, with the aim of reprogramming the TME, initiating an endogenous anti-tumour immune response and/or directly acting on CAR T cells to improve their function. Numerous transgenes have been tested in an armoured CAR T cell setting, however this review will focus on IL-12 and IL-18, which have been studied the most extensively.

IL-12 Armoured CAR T Cells

IL-12 is often considered to be the frontrunner in the armoured CAR T cell field, as the first CAR T cell "armour" to be tested in a clinical setting. Physiologically, IL-12 is produced by macrophages, monocytes, DCs, granulocytes and B cells, and has the primary functions of enhancing the cytotoxic activity of CD8+ T cells and NK cells, and facilitating CD4+ T cell differentiation to the pro-inflammatory, anti-tumour Th1 phenotype (Zundler and Neurath, 2015). Early studies in mouse models demonstrated that intraperitoneal administration of recombinant IL-12 suppressed tumour growth, which was predominantly associated with CD8+ T cell activity, with contribution from other IL-12 induced mechanisms such as increased T cell and NK cell infiltration, IFN-γ production, IFN-γ-induced chemokines CXCL9 and CXCL10, and NK cell activation (Brunda et al., 1993; Cavallo et al., 1999; Colombo et al., 1996; Tahara et al., 1996). However, the severe toxicities observed in subsequent clinical trials (Leonard et al., 1997) led to exploration of more localised forms of IL-12 delivery, such as through ACT. Overexpression of IL-12 in ACT has been tested in TILs (Zhang et al., 2015a), TCR-modified T cells (Kerkar et al., 2010, 2011; Zhang et al., 2011, 2020a) and CAR T cells (Chinnasamy et al., 2012; Chmielewski et al., 2011; Koneru et al., 2015b, 2015a; Pegram et al., 2012; Yeku et al., 2017), demonstrating improved tumour control upon treatment with IL-12-secreting T cells. Interestingly, endogenous T and NK cells do not seem to be the key mediators of this improved anti-tumour response, as therapeutic efficacy was unaffected in studies utilising T and NK cell-deficient mice (Chinnasamy et al., 2012; Chmielewski et al., 2011; Kerkar et al., 2011; Yeku et al., 2017). Rather, multiple studies have indicated a role of the myeloid compartment in mediating the anti-tumour activity of IL-12-expressing T cells. Collectively, studies point towards a model where IL-12-secreting T cells deplete suppressive tumour-infiltrating immune cells including MDSCs and TAMs (Chinnasamy et al., 2012; Kerkar et al., 2011; Yeku et al., 2017), reprogram TAMs and DCs to have improved antigen presentation capabilities and enhance TNF secretion by TAMs (Chmielewski et al., 2011; Kerkar et al., 2011), enhancing the function of adoptively transferred T cells in an indirect manner. Studies have also demonstrated that the secreted IL-12 improves CAR T

cells through autocrine IL-12 signalling and the resulting increase in IFN-γ production is important for efficacy (Kerkar et al., 2011; Pegram et al., 2012). Furthermore, there is some indication that IL-12-expressing CAR T cells are resistant to Treg-mediated and PD-L1-mediated inhibition (Pegram et al., 2012; Yeku et al., 2017).

IL-18 Armoured CAR T Cells

IL-18 is a pleiotropic cytokine which acts on a range of immune cells, including T cells, NK cells, DCs and macrophages, to stimulate their production of IFN-γ (Yasuda et al., 2019). Notably, IL-12 and IL-18 often function together, as IL-12 stimulates expression of the IL-18 receptor on T cells, allowing them to respond to IL-18 signalling (Nakahira et al., 2001; Tomura et al., 1998). Like IL-12, recombinant IL-18 has been assessed in clinical trials as a cancer therapeutic (Robertson et al., 2006, 2013). However, while the resulting toxicities were mild, the therapeutic efficacy was limited, although serum analyses showed increased activation of lymphocytes and serum IFN-γ concentration. IL-18 CAR T cells have been shown to exhibit improved IFN-γ production, proliferation and persistence *in vivo*, dependent on autocrine signalling and, interestingly, signalling through their endogenous TCR (Avanzi et al., 2018a; Hu et al., 2017). Modulation of the TME by IL-18 CAR T cells has also been demonstrated, leading to expansion of endogenous NK cells, NKT cells, DCs, CD8+ T cells, reprogramming of macrophages and DCs to a more pro-inflammatory phenotype, and depletion of Tregs, suppressive macrophages and DCs (Avanzi et al., 2018a; Chmielewski and Abken, 2017). Treatment with IL-18 CAR T cells has also been shown to eliminate antigen-negative tumour cells (Avanzi et al., 2018b).

Limitations of Armoured CAR T Cells

Toxicities

Despite promising outcomes in preclinical models, the use of armoured CAR T cells in the clinic has been limited by severe toxicities. This was demonstrated in a previous clinical trial utilising IL-12-secreting TILs for the treatment of metastatic melanoma, where high cell doses led to life-threatening toxicities including liver dysfunction, high fevers and haemodynamic instability (Zhang et al., 2015b).

Additionally, current methods to introduce transgenes into T cells involve the use of retroviral vectors, which result in random integration of the transgene into the genome at varying frequencies. While toxicities related to random transgene integration have not yet been observed in the context of armoured CAR T cells, this introduces a further risk for clinical translation. Importantly, this limitation highlights the need to develop advanced genome engineering strategies to induce localised

expression of pro-inflammatory transgenes, which is the subject of the current project. This approach also has the advantage of allowing for a wider range of genes to be expressed by the CAR T cells, including those that require a concentration gradient to be effective, such as the chemokines CXCL9 and CXCL10.

Section 3: CRISPR/Cas9 Genome Engineering

CRISPR/Cas9 genome engineering offers a strategy to fine tune genetic circuitry through the ability to target specific DNA sequences. CRISPR, which stands for clustered regularly interspaced short palindromic repeats, functions in prokaryotes as a defence mechanism against bacteriophages (Lander, 2016). This consists of a series of DNA sequences in prokaryotic genomes that encode DNA from various bacteriophages, which, when transcribed into RNA, function as a complex with an endonuclease to cleave the DNA of invading bacteriophages. While there are several different variations of CRISPR systems that have been discovered, the most commonly used system for gene editing purposes is the Type II system from *Streptococcus pyogenes*, which utilises the endonuclease Cas9 (CRISPR-associated protein 9) (Lander, 2016).

CRISPR/Cas9 gene editing enables targeted modifications to the genome through the introduction of a double strand break (DSB) at the target site, mediated by Cas9 endonuclease complexed with a target-specific guide RNA. The guide RNA is often introduced as a single construct, or single-guide RNA (sgRNA), consisting of a trans-activating CRISPR RNA sequence which enables complex formation with Cas9, and the target-specific CRISPR RNA. Following the induction of a DSB, endogenous DNA repair mechanisms would perform non-homologous end joining (NHEJ) to repair the DSB, leading to the introduction of insertions and deletions at the DSB site, often resulting in gene disruption (CRISPR knockout). In the presence of an exogenous DNA repair template containing desired modifications to the genome flanked by homologous sequences to the target site, the DSB would be repaired by homology-directed repair (HDR) using the repair template, leading to modifications in the target gene.

3.1 - CRISPR/Cas9 Editing in T Cells

The use of CRISPR/Cas9 gene editing in mammalian T cells was made possible after several key developments in the delivery of CRISPR components. Delivery of transgenes into primary T cells is conventionally performed using retroviral or lentiviral vectors (Bunnell et al., 1995; Costello et al., 2000), and remains to be the most commonly used strategy for CAR T cell transduction. However, generation of such viral vectors is costly and labour-intensive, and the size and number of constructs that can be packaged using viral vectors is limited, preventing this strategy from being used for delivering CRISPR components. Thus, the first successful application of CRISPR in T cells utilised

transfection of Cas9- and sgRNA-encoding plasmids into cells by electroporation (Mandal et al., 2014). However, gene disruption was only achieved in 1 – 5% of T cells. This was significantly improved with the use of recombinant Cas9 complexed with sgRNA, achieving 40% gene disruption when tested in T cells for the first time (Kim et al., 2014; Schumann et al., 2015).

Delivering the HDR repair template for introducing directed modifications to the T cell genome has also posed a challenge in T cells. Early studies successfully utilised single-stranded oligonucleotides (ssODNs) or double-stranded DNA (dsDNA) to deliver repair templates into cells (Roth et al., 2018; Schumann et al., 2015). However, low integration efficiencies were observed with larger insert sizes, and exogenous dsDNA is well established to be toxic to cells (Hornung and Latz, 2010; Roth et al., 2018). Therefore, applications of CRISPR in CAR T cells have utilised adeno-associated viral vectors (AAV) to introduce the HDR repair template into T cells (Dai et al., 2019; Eyquem et al., 2017; MacLeod et al., 2017; Wiebking et al., 2020). AAV is a well-established viral vector for the delivery of exogenous DNA into cells, and there are currently numerous clinical trials assessing the use of AAV for gene therapies as well as ACT for cancer treatment (Naso et al., 2017; Wang et al., 2020). AAV is a favourable viral vector for CRISPR-HDR applications as its single stranded genome is stabilised by terminal hairpins, enabling its persistence as an episome inside host cells (Wang et al., 2020).

3.2 - CRISPR in CAR T Cells

The ability to introduce specific and precise edits into the genome using CRISPR/Cas9 technology has created new avenues for improving CAR T cell design, and several groups have started to explore this potential. To combine the effects of immune checkpoint inhibition with CAR T cell therapy, several studies have demonstrated that knocking out PD-1 in human CAR T cells leads to improved efficacy in tumour xenograft models (Ren et al., 2017; Rupp et al., 2017). Ren et al. also simultaneously disrupted the endogenous TCR and HLA-I loci, with the aim of addressing the need to develop off-the-shelf CAR T cell products, and successfully demonstrated reduced alloreactivity. Importantly, this study was the first to demonstrate efficient multiplex CRISPR knockout in human CAR T cells (Ren et al., 2017). Another issue in CAR T cell generation is the random integration of the CAR construct into the T cell genome, posing the risk of oncogenic transformation, gene disruption and variation in CAR expression between transduced cells (Ellis, 2005; von Kalle et al., 2014). To address this, one study replaced the TRAC gene in human T cells with a CD19-specific CAR, resulting in uniform CAR expression as well as improved anti-tumour function, as the CAR was under TCR-specific regulation, leading to delayed T cell exhaustion (Eyquem et al., 2017). Extending from this work, a recent study demonstrated the possibility of multiplex gene insertion using CRISPR-HDR, by respectively targeting the PDCD1 and TRAC locus to generate CD19 and

CD22 dual-specific CAR T cells, in a knock in-knock out fashion (Dai et al., 2019). Excitingly, a recent first-in-human phase I clinical trial has demonstrated the use of autologous T cells transduced with a NY-ESO-1-specific TCR, with CRISPR-mediated disruption of TCRα, TCRβ and PD-1 to treat three patients with advanced, refractory cancer (Stadtmauer et al., 2020). This study reported successful engraftment, mild toxicities and persistence of the CRISPR-engineered T cells for up to 9 months (Stadtmauer et al., 2020). Although this clinical trial utilised T cells expressing a transgenic TCR rather than a CAR, these results indicate the future potential for CRISPR-engineered CAR T cell therapy.

3.3 - The Use of CRISPR for Generating Armoured CAR T Cells

While studies thus far have been focused on using CRISPR-HDR to insert the CAR into the T cell genome, another possible application of this technique is for the generation of armoured CAR T cells, which is the aim of my PhD project. CRISPR may offer the flexibility and regulatory control required to improve armoured CAR T cell design, in terms of achieving tumour-specific expression of the transgene with minimal toxicity. To achieve this, the transgene could be inserted into the genome under the control of an endogenous promoter that is highly expressed in the tumour, but not in peripheral tissues. Furthermore, in selecting a suitable promoter, the regulatory mechanisms that control T cell function could be exploited to further boost CAR T cell activity; for example, genes that regulate CAR T cell exhaustion would be expected to be highly upregulated specifically at the tumour site. Our armouring approach will therefore involve identifying inhibitory genes that have high tumour-specific expression, and replacing these genes with pro-inflammatory genes using CRISPR-HDR. This would have the two-fold benefit of achieving tumour-specific expression of the transgene, and the simultaneous disruption of an inhibitory gene (Figure 1).

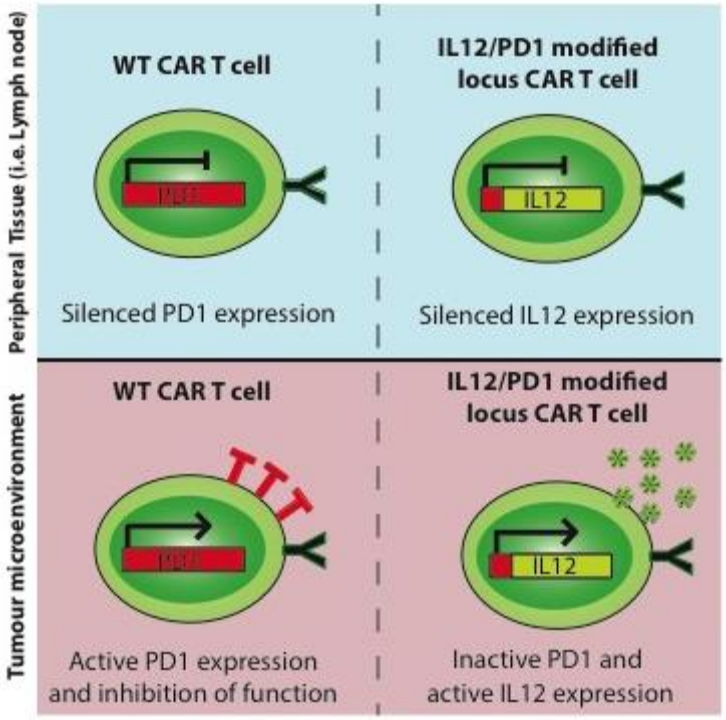

Figure 1. Schematic of CRISPR-HDR strategy to engineer CAR T cells to express pro-inflammatory cytokines exclusively at the tumour site, using PD-1 and IL-12 as an example.

3.3.1 - Potential Targets

RNA sequencing data generated using our syngeneic Her2 CAR T cell mouse model (Lauren Giuffrida, Beavis laboratory) has revealed a number of potential target genes that exhibit differential expression in the tumour compared to the spleen.

PD-1

The clinical efficacy of anti-PD-1 checkpoint blockade therapy is well documented (Ribas and Wolchok, 2018), and has been shown to improve CAR T cell therapy when delivered in combination (Cherkassky et al., 2016; John et al., 2013; Serganova et al., 2017; Wang et al., 2019) or through CRISPR-mediated PD-1 disruption (Ren et al., 2017; Rupp et al., 2017). Therefore, replacing PD-1 in CAR T cells with a pro-inflammatory gene through our CRISPR-HDR approach will be clinically relevant, and likely to confer improved therapeutic efficacy.

NR4A Transcription Factors

The NR4A transcription factor family consists of three members, NR4A1, NR4A2 and NR4A3, which were recently identified as key regulators of T cell exhaustion (Chen et al., 2019a; Liu et al., 2019). Specifically, Chen et al. demonstrated that CAR T cells deficient in all three NR4A transcription factors had improved effector function and enhanced tumour control. Our RNA-seq data

suggest that all three members of the NR4A family have high differential expression in the tumour compared to the spleen, with NR4A2 exhibiting the greatest increase in expression.

3.3.2 - Potential Transgenes

Tumour necrosis factor (TNF)

TNF is an inflammatory cytokine that can mediate direct killing of tumour cells through binding to the TNF receptor, and is a key anti-tumour effector utilised by CAR T cells (Balkwill, 2009; Kearney et al., 2018; Michie et al., 2019). Importantly, both mouse and human CD8+ T cells have been demonstrated to downregulate TNF expression in their exhausted or dysfunctional state following chronic TCR signalling in the TME (Fourcade et al., 2010; Philip et al., 2017). Therefore, replacing an exhaustion gene with TNF in CAR T cells may help to maintain TNF expression over time.

CXCL9, CXCL10 and CXCL11

As outlined above, CXCL9, CXCL10 and CXCL11 are the key chemokines responsible for mediating T cell trafficking into tumours (House et al., 2020; Mikucki et al., 2015). The localised production of these chemokines in the TME may assist in improved recruitment of CAR T cells and endogenous anti-tumour immune cells.

Type I Interferons

Type I interferons (IFN), consisting of IFNα and IFNβ, play a fundamental role in mediating anti-tumour immune responses through eliciting widespread effects on immune cells (Borden, 2019), such as through enhancing recruitment of cross-presenting DCs into tumours (Fuertes et al., 2016). Type I IFN production in the TME has been shown to improve the therapeutic efficacy of immunotherapies (Bald 2014) as well as chemotherapy (Sistigu 2014) and radiotherapy (Burnette 2011). Hence, local production of type I IFNs by CAR T cells may help to engage endogenous immune mechanisms and promote *de novo* anti-tumour immune responses.

Section 4: Project hypotheses and aims

Hypothesis:

Engineering CAR T cells to express immunomodulatory factors such as cytokines and chemokines under the control of endogenous promoters with tumour-specific expression will enable them to overcome tumour-induced immunosuppression and enhance their therapeutic efficacy.

Aims:

1. Develop a CRISPR-HDR approach to integrate genes into the genome of mouse and human T cells.
   a) Assess the feasibility of CRISPR-HDR in mouse and human T cells.
   b) Optimise a CRISPR-HDR workflow to achieve maximal HDR efficiency in:
      - OT-I cells
      - Mouse CAR T cells
      - Human CAR T cells

2. Identify potential target loci and transgenes to be investigated.
   a) Potential target loci: Perform RNA sequencing to identify genes that have high differential expression in the tumour.
   b) Potential transgenes: Identify genes that have been demonstrated to contribute to the anti-tumour immune response, based on previous work from our lab or from the literature.

3. Assess the therapeutic efficacy and interrogate the immunological mechanism and safety of armoured CAR T cells (in both human and mouse systems) generated by the CRISPR/Cas9 engineering approach developed in Aim 1, testing several target loci/transgene combinations determined in Aim 2.

Summary of Progress

Aim 1a: Assess the feasibility of CRISPR-HDR in mouse and human T cells.

Genomic integration of large gene fragments by CRISPR-HDR has not been previously demonstrated in primary mouse T cells, and has only been reported in primary human T cells in a small number of studies (Dai et al., 2019; Eyquem et al., 2017; MacLeod et al., 2017; Roth et al., 2018; Stadtmauer et al., 2020). Therefore, we sought to assess the feasibility and CRISPR-HDR editing efficiency in primary mouse and human T cells. A repair template to integrate a GFP transgene at the start of the PD-1 locus, which would concurrently disrupt the PD-1 gene, was designed for both mouse and human genomes (Figure 2A). Briefly, this consisted of a GFP transgene flanked by 350 base pair (bp) homology arms directly upstream and downstream of the PD-1 transcription start site. A 3' poly-A tail was included in the GFP transgene sequence to stabilise the GFP transcript, and in combination with the stop codon at the end of the GFP sequence, these elements would prevent the transcription and translation of PD-1. Furthermore, the PAM sequence, which enables Cas9 to induce a DSB, was mutated in the repair template to prevent cleavage by Cas9. An addition of one bp at the start of the 3' homology arm was also introduced to cause a frameshift mutation in PD-1, in the case that PD-1 is still transcribed from an edited locus, although the likelihood of this is low. An sgRNA targeting the start of the PD-1 transcription start site was designed to enable insertion of the GFP transgene.

For initial experiments, mouse and human T cells were edited following the workflow illustrated in Figure 2B, with appropriate adjustments based on the specific cells. In general, human T cells were obtained through isolating PBMCs from a buffy coat and culturing with an αCD3 antibody and IL-2 for 72 hours to selectively expand T cells. Mouse T cells were obtained by harvesting and processing spleens from mice, and culturing with αCD3, αCD28, IL-2 and IL-7 to expand T cells. T cells were electroporated to introduce Cas9, PD-1 sgRNA and the repair template in the form of a PCR-amplified dsDNA product into the cells. T cells were assessed for HDR editing efficiency based on GFP expression by flow cytometry after 3 – 4 days in culture.

T cells from two mouse models in this project: T cells from C57BL/6 mice, which are routinely used for generation of mouse CAR T cells in our laboratory, and T cells from transgenic OT-I mice (C57BL/6 background), which possess a TCR specific to the ovalbumin peptide SIINFEKL. The latter model enables the rapid expansion of T cells *in vitro* due to the ability to stimulate OT-Is with recombinant SIINFEKL, and allows the assessment of anti-tumour efficacy of CRISPR-edited T cells without an additional CAR transduction procedure. Hence, the OT-I system is an ideal model for proof-of-concept CRISPR-HDR experiments, that also maintains clinical relevance to TCR-modified T cell therapy.

CRISPR-HDR editing with the PD-1/GFP repair template in OT-I T cells resulted in editing efficiencies of approximately 5% (Figure 2C). Similar editing efficiencies were seen in human T cells (data not shown); however, as human T cells are more permissive to long term maintenance *in vitro*, GFP positive T cells could be sorted by FACS to obtain a purer population of edited cells (Figure 2D). However, this only resulted in a GFP positive population of 15%, possibly due to the low PD-1 expression on the T cells at the time of analysis, or the outgrowth of unedited T cells which may have been inadvertently collected during the FACS procedure. Nevertheless, these data represent the first instances of CRISPR-HDR editing in primary T cells in our laboratory, and future experiments are aimed at improving the editing efficiency and workflow utilised to produce functional edited T cells for both *in vitro* and *in vivo* experiments.

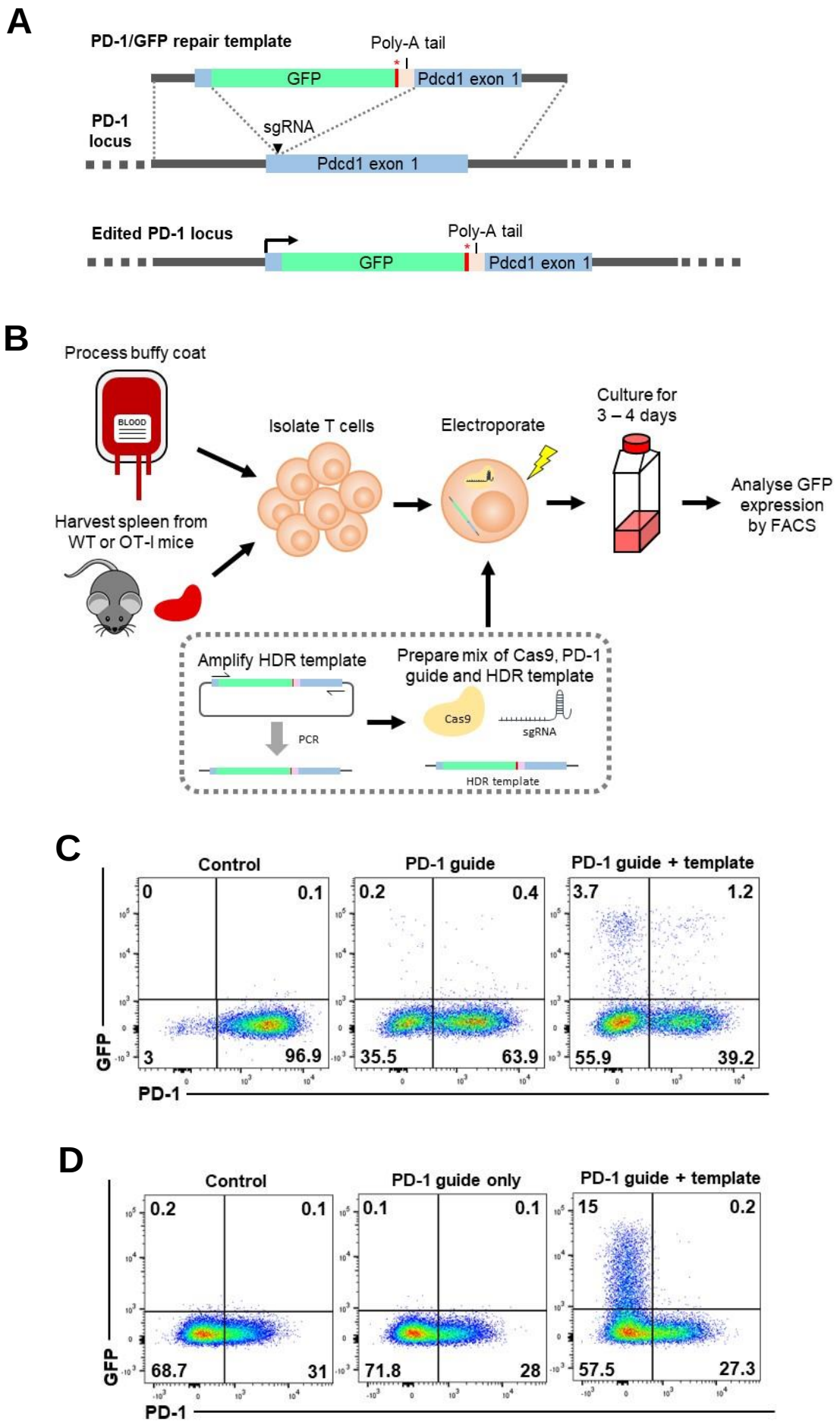
A
PD-1/GFP repair template
Poly-A tail
GFP
Pdcd1 exon 1
PD-1 locus
sgRNA
Pdcd1 exon 1
Edited PD-1 locus
Poly-A tail
GFP
Pdcd1 exon 1
B
Process buffy coat
BLOOD
Isolate T cells
Electroporate
Culture for 3 – 4 days
Analyse GFP expression by FACS
Harvest spleen from WT or OT-I mice
Amplify HDR template
PCR
Prepare mix of Cas9, PD-1 guide and HDR template
Cas9
sgRNA
HDR template
C
Control
PD-1 guide
PD-1 guide + template
0
0.1
3
96.9
0.2
0.4
35.5
63.9
3.7
1.2
55.9
39.2
GFP
PD-1
D
Control
PD-1 guide only
PD-1 guide + template
0.2
0.1
68.7
31
0.1
0.1
71.8
28
15
0.2
57.5
27.3
GFP
PD-1

Figure 2. CRISPR-HDR editing of the PD-1 locus in human and mouse T cells using a dsDNA repair construct. (A) Schematic of PD-1/GFP repair template designed to disrupt genomic PD-1 expression through CRISPR-HDR. (B) General workflow utilised to edit human and mouse T cells by CRISPR-HDR. (C-D) Representative plots of CRISPR-HDR editing efficiency in OT-I (C) and human (D) T cells. A guide targeting an unrelated gene was utilised as the control. In (C), naïve OT-I T cells were electroporated and cultured with 10 nM SIINFEKL peptide for 3 days to induce activation and proliferation, leading to high PD-1 expression. HDR efficiency was assessed 3 days after electroporation. In (D), human PBMCs isolated from a buffy coat were activated with an anti-CD3 antibody and IL-2 to expand T cells, before electroporating and culturing for 3 days. GFP-expressing T cells were sorted by FACS, and cells were cultured for an additional 3 days before analysing GFP expression by flow cytometry.

Aim 1b: Optimise a CRISPR-HDR workflow to achieve maximal HDR editing efficiency.

Following the initial proof-of-concept experiments in mouse and human T cells, subsequent experiments have been focused on improving HDR editing efficiency in mouse T cells. Following a literature search, a number of various strategies reported to improve HDR efficiency were assessed, including the activation of T cells prior to electroporation (Lin et al., 2014), and the use of small molecule inhibitors to inhibit NHEJ machinery (Chu et al., 2015; Maruyama et al., 2015; Srivastava et al., 2012) or stabilise Cas9/sgRNA complexes prior to electroporation (Nguyen et al., 2019). However, these strategies did not improve HDR editing efficiency in primary mouse T cells (data not shown).

Instead, the use of an AAV6 vector to deliver the HDR template, which has been successfully used in human T cells (Dai et al., 2019; Eyquem et al., 2017; MacLeod et al., 2017; Wiebking et al., 2020), was able to drastically improve editing efficiencies in WT mouse T cells (Figure 3). AAV vectors are efficient at supporting CRISPR-HDR editing for various reasons (refer to Section 3.1 of literature review). An AAV6 vector containing the PD-1/GFP repair template was purchased from a commercial vendor, and used to transduce WT mouse T cells according to a protocol established by our collaborators in Stanford University (Porteus laboratory). Briefly, mouse T cells are added to prepared AAV6 solutions in a 96 well plate immediately after electroporation to facilitate "electroporation-aided transduction", where it is hypothesised that the pores formed in the cell membrane during electroporation assists in AAV6 infection. Following CRISPR-HDR editing, cells are cultured for one week to minimise episomal expression from the AAV6 genome, which can be observed in the "AAV only" controls. Stimulating the TCR of cells with plate-bound αCD3 and αCD28 upregulates PD-1 expression, which would induce edited cells to produce GFP. This protocol has led to an HDR editing efficiency of 40%, which is sufficient for biological experiments, although it is likely that higher editing efficiencies can be achieved through further modifications to the protocol.

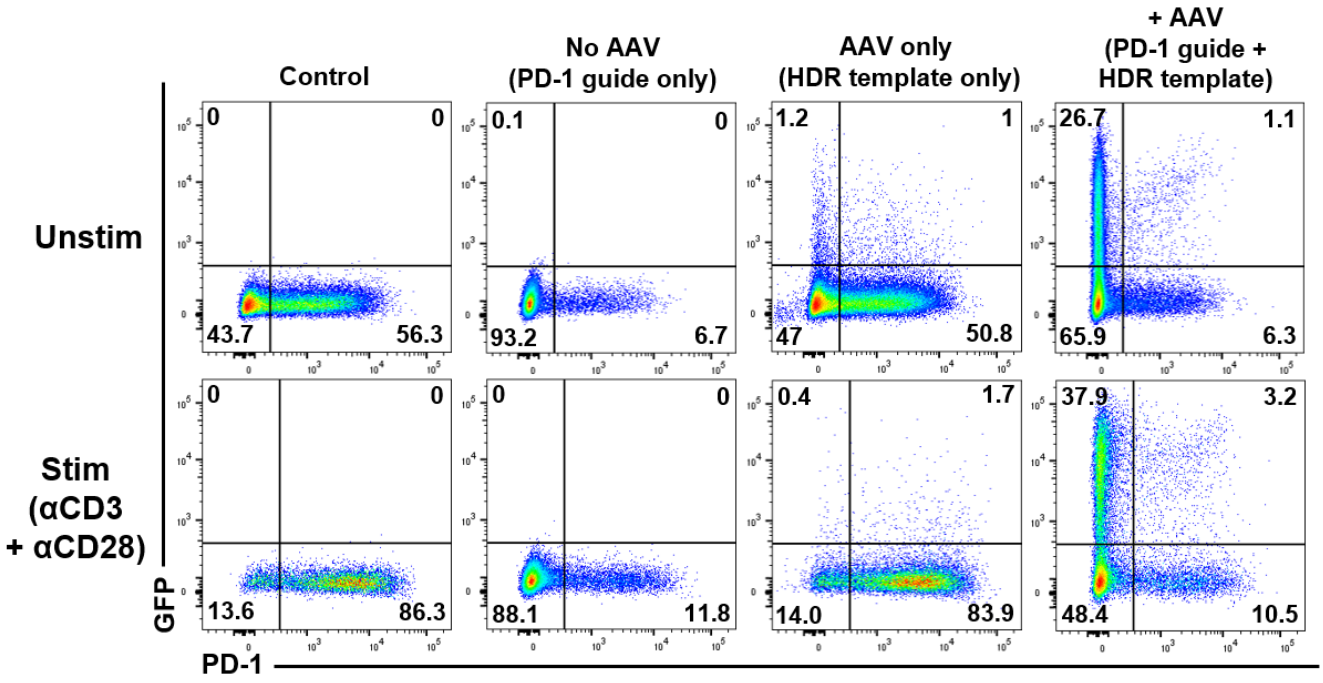

Figure 3. Delivering HDR repair templates using an AAV vector dramatically improves HDR editing efficiency. WT mouse T cells were electroporated with Cas9 and PD-1 sgRNA, and incubated with PD-1/GFP AAV6 at 200K MOI for 3 days. Cells were cultured for an additional 4 days, before stimulation with plate-bound αCD3 and αCD28 for 24 hours to upregulate PD-1 expression. GFP expression was analysed by flow cytometry. "Control" and "AAV only" refers to cells electroporated without Cas9 and PD-1 guide, and cultured without or with AAV6, respectively. "No AAV" refers to cells electroporated with Cas9 and PD-1 guide, but cultured without AAV6.

Aim 2a: Identify potential target loci to be investigated.

PD-1 was chosen as the target for initial experiments due to its clinical relevance; however, it is possible that other genes with high differential expression in the tumour may function as better targets in our CRISPR-HDR system. Hence, RNA-seq was performed to compare gene expression in mouse CAR T cells in the tumour compared to the spleen (Figure 4), to identify other potential gene targets. Indeed, PD-1 exhibits high differential expression in CAR T cells in the tumour compared to the spleen. However, the RNA-seq data suggests that there are other genes which exhibit greater differential expression in the tumour, most of which are either novel or play known roles in tumour cell killing (eg. Granzyme B). Notably, the recently discovered transcription factor NR4A2 (refer to Section 3.3.2 in literature review) is differentially expressed in the tumour and is more highly expressed than PD-1. Hence, we aim to assess NR4A2 as an additional target in our CRISPR-HDR system.

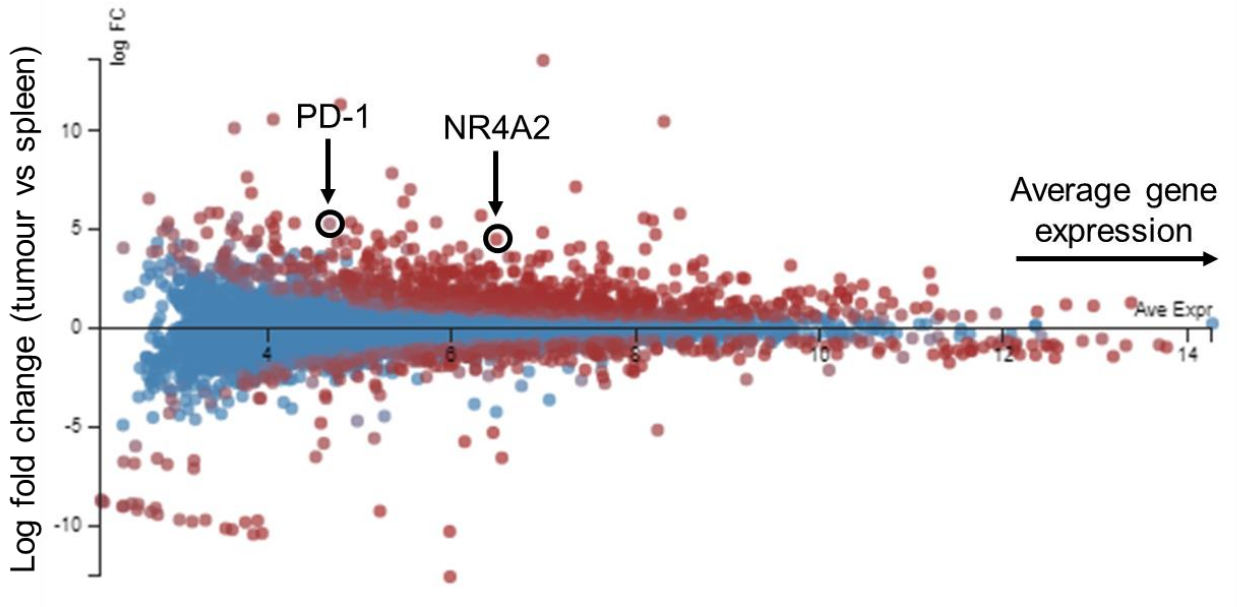

Figure 4. Differential expression of genes in αHer2 CAR T cells in the tumour compared to the spleen. Transgenic Her2 mice were inoculated with 0.2 x 10^6 E0771-Her2 tumour cells in the mammary fat pad. On days 6 and 7 after tumour injections, mice were treated with 20 x 10^6 anti-Her2 CAR T cells, administered in two doses of 10 x 10^6 cells per day. Tumours and spleens were harvested from mice 8 days following CAR T cell treatment, and CAR T cells were isolated by fluorescence activated cell sorting (FACS). 3' RNA-seq was performed and data was analysed on Degust. Data shown is from n = 2 biological replicates.

As our CRISPR-HDR armoured CAR T cell system will involve the replacement of PD-1 or NR4A2 with a pro-inflammatory transgene, the viability and phenotype of PD-1 and NR4A2 KO CAR T cells *in vivo* should first be assessed. Thus, PD-1 and NR4A2 KO αHer2 CAR T cells were generated and assessed in our syngeneic Her2 transgenic mouse model. Briefly, our Her2 transgenic mouse model expresses Her2 in certain tissues, enabling engraftment of Her2-expressing tumours without Her2-mediated rejection by the host immune system.

PD-1 and NR4A2 disruption in αHer2 CAR T cells led to similar transduction efficiencies (Figure 5A), assessed by myc tag expression, which is fused to the Her2 CAR construct, and NGFR expression, which is cotransduced into T cells alongside the Her2 CAR vector. PD-1 and NR4A2 KO CAR T cells appear to have decreased engraftment in the tumour and spleen compared to mock KO CAR T cells, although this difference was not statistically significant (Figure 5B). In terms of cytotoxicity, both PD-1 and NR4A2 KO CAR T cells seem to have improved IFN-γ production, with NR4A2 KO CAR T cells also exhibiting improved granzyme B production following restimulation *ex vivo*, albeit statistically non-significant (Figures 5C-D). NR4A2 KO CAR T cells express higher levels of Ki67 compared to mock KO CAR T cells, suggesting a greater proliferation capacity (Figure 5E). PD-1 KO CAR T cells exhibit significantly lower PD-1 expression compared to the mock KO CAR T cells in the tumour, which is consistent with genomic disruption of PD-1 (Figure 5F). Although statistically non-significant, PD-1 appears to have elevated Tim3 expression in the tumour compared to mock KO CAR T cells (Figure 5G). NR4A2 KO CAR T cells have significantly increased expression of both PD-1 and Tim3 in the tumour compared to mock KO CAR T cells, indicative of improved effector functions (Figure 5F-G). In terms of effector/memory phenotype, both PD-1 and NR4A2 KO CAR T cells appear to have an increased proportion of effector memory T cells in the tumour based on the CD62L- CD44+ population, which is correspondingly decreased in the spleen, although only the NR4A2 KO data is statistically significant (Figure 5H). There is some indication that PD-1 and NR4A2 KO CAR T cells have a decreased frequency of central memory phenotype T cells (Figure 5I). Lastly, a lower proportion of stem-like memory NR4A2 KO CAR T cells was observed in the tumour, with a corresponding increase in the spleen (Figure 5J). Altogether, these data suggest that PD-1 and NR4A2 KO αHer2 CAR T cells are able to achieve successful engraftment in our syngeneic Her2 transgenic mouse model, and, particularly for NR4A2 KO CAR T cells, possess a more effector-like phenotype in terms of cytokine production, expression of exhaustion markers and effector/memory markers, which is consistent with the literature (Chen et al., 2019a; Ren et al., 2017; Rupp et al., 2017). Future experiments will assess the therapeutic efficacy of PD-1 and NR4A2 KO CAR T cells *in vivo*, before targeting these loci in our CRISPR-HDR armoured CAR T cell strategy.

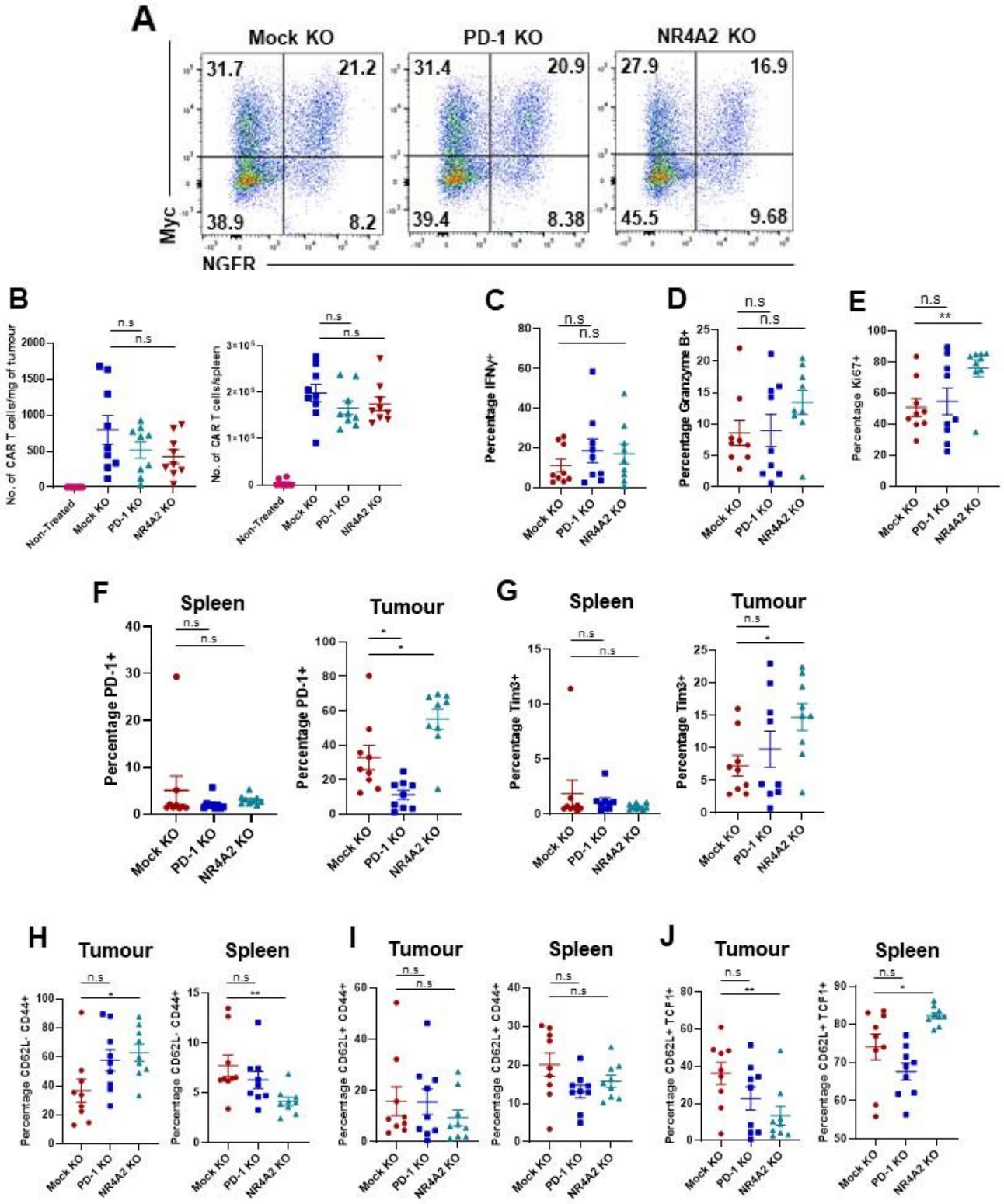

Figure 5. Phenotype and persistence of PD-1 and NR4A2 KO αHer2 CAR T cells *in vivo*. Spleens from WT donor mice were harvested, processed and electroporated with Cas9 and either PD-1, NR4A2 or a non-targeting control guide (Mock KO), then activated overnight with αCD3, αCD28, IL-2 and IL-7 to selectively expand T cells. T cells were transduced with an αHer2 CAR using a retroviral vector, and injected intravenously into mice bearing E0771-Her2 tumours in the mammary fat pad 6 days following tumour inoculation and donor spleen activation. (A) αHer2 CAR

transduction efficiency was assessed prior to injection. 7 days after CAR T cell treatment, tumours and spleens were harvested and stained for various markers for flow cytometry analyses. As CAR expression is downregulated upon recognition of CAR targets *in vivo*, CAR T cells were identified as CD8+ NGFR+ cells. (B) Number of CAR T cells in the tumour, relative to tumour mass, and per spleen. (C – E) Tumours were restimulated with PMA, ionomycin and Golgi plug/stop to stimulate cytokine production, and intracellular staining was performed to assess expression of (C) IFN-γ, (D) granzyme B and (E) Ki67. Unstimulated tumours and spleens were stained for (F) PD-1, (G) Tim3, and various markers to assess effector/memory phenotypes, notably (H) CD62L- CD44+ to indicate effector memory T cells, (I) CD62+ CD44+ to indicate central memory T cells and (J) CD62L+ TCF1+ to indicate stem-like memory T cells. Data shown is from n = 9 mice/group. Statistical significance between the mock KO and either PD-1 KO or NR4A2 KO groups was calculated using an unpaired t test. * $p < 0.05$, ** $p < 0.01$.

Aim 3: Assess the therapeutic efficacy of armoured CAR T cells generated by CRISPR-HDR.

A preliminary experiment to assess the tumour killing capacity of armoured CAR T cells generated by our CRISPR-HDR strategy has been performed. A PD-1/TNF repair template was generated by replacing GFP in the PD-1/GFP template with a TNF transgene. For these experiments, a dsDNA repair template was used to edit OT-I T cells, following the workflow outlined in Figure 2B. Editing efficiency was assessed by flow cytometry (Figure 6A), by treating cells with Golgi plug/stop for 4 hours to prevent secretion of TNF, and by cytometric bead array (Figure 6B) to assess TNF concentration in cell supernatants following 3 days in culture. Edited cells exhibited a slight increase in TNF production, which is consistent with the low editing efficiency when dsDNA repair templates are used (Figure 2C). However, this was sufficient to elicit an observable improvement in tumour cell killing *in vitro*, assessed through an overnight chromium killing assay (Figure 6C). Briefly, this assay involves the coculture of T cells with tumour cells labelled with radioactive chromium-51 (^{51}Cr), and tumour cell killing is measured by the amount of ^{51}Cr released by dying target cells. Edited OT-I T cells exhibited improved killing of antigen-negative MC38 parental cells, which do not express the ova antigen, suggesting that their production of TNF was sufficient to mediate bystander killing of tumour cells (Balkwill, 2009; Kearney et al., 2018; Michie et al., 2019). The similar extent of ^{51}Cr release in the MC38 ova cultures are likely due to high levels of TCR-mediated killing by both unedited and edited OT-I T cells, overpowering the additional TNF-dependent anti-tumour effects from the edited OT-I T cells. Given that HDR editing with an AAV6 vector is able to improve integration efficiency to ~40%, utilising an AAV6 vector to introduce the PD-1/TNF template in future experiments is likely to result in a more significant enhancement of tumour cell killing.

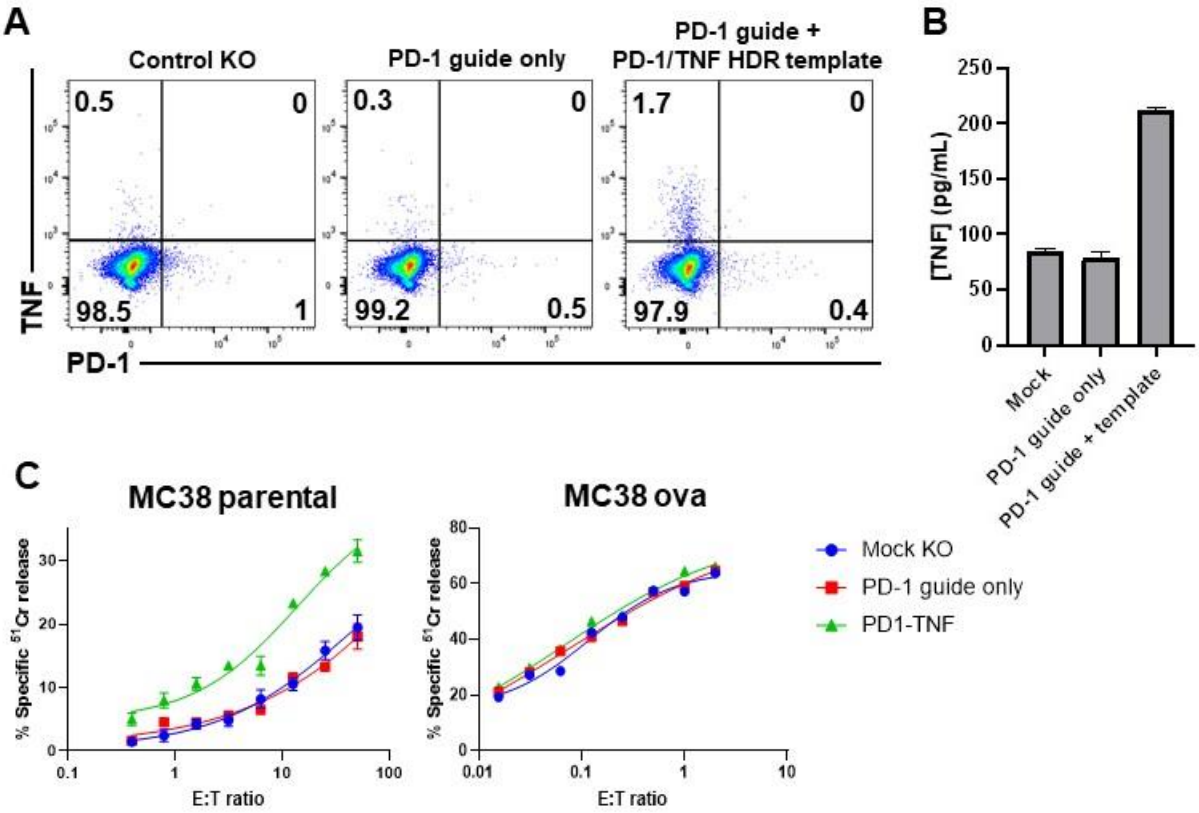

Figure 6. Expression of TNF from the PD-1 locus in OT-I T cells leads to improved tumour cell killing *in vitro*. OT-I T cells were edited by CRISPR-HDR using a PD-1 sgRNA and a PD-1/TNF dsDNA repair template, to integrate TNF into the PD-1 locus. Knock-in efficiency was assessed by (A) flow cytometry through intracellular staining for TNF, and (B) cytometric bead array to assess TNF concentration in cell supernatants. (C) Edited OT-I T cells were assessed for tumour killing capacity through an overnight chromium killing assay, against MC38 parental (antigen negative) or MC38 ova (antigen positive) targets. Data shown is from n = 1 experiments.

In conclusion, I have developed a CRISPR-HDR methodology to integrate large gene fragments into the mouse T cell genome, identified suitable gene targets for our armouring strategy and validated that disruption of these genes does not affect CAR T cell engraftment in our Her2 transgenic mouse model. Subsequent experiments will involve the use of CRISPR-HDR with AAV6 to engineer mouse T cells to express TNF, CXCL9/10 or type I IFNs from either the PD-1 or NR4A2 gene locus, and assess their phenotype and functions *in vitro* and *in vivo*.

Research Plan

Aims		Progress	2019					2020							2021											2022						
			First year												Second year											Third year						
			Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul
Aim 1: Develop CRISPR-HDR methodology	Aim 1a: Assess feasibility of CRISPR HDR	Complete																																		
	Aim 1b (i): Optimise CRISPR HDR in OT-Is	Almost complete																																		
	Aim 1b (ii): Optimise HDR in mouse CAR T cells	Almost complete																																		
	Aim 1b (iii): Optimise HDR in human CAR T cells	Not started																																		
Aim 2: Identify potential target loci and transgenes	Aim 2a: RNA sequencing to determine target loci	Complete																																		
	Aim 2b: Identify potential transgenes	First stage complete	Identify transgenes for immediate investigation												Ongoing evaluation of potential transgenes based on literature																						
Aim 3: Assess therapeutic efficacy of armoured CAR T	Aim 3a: In mouse OT-I cells and CAR T cells	About to commence																																		
	Aim 3b: In human CAR T cells	Not started																																		

Grey = Complete
Orange = Ongoing
Blue = Not started/about to commence

Research Outputs

Publications:

Chen A. X. Y., House I. G., Beavis P. A., Darcy P. K. A New Safety Approach Allowing Reversible Control of CAR T Cell Responses. *Mol Ther*. 2020;28(7):1563-1566. doi:10.1016/j.ymthe.2020.06.009

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