

# Dissecting the impact of Chimeric Antigen Receptor expression levels on T cell behaviour and response



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# Abstract

Chimeric antigen receptor (CAR) T cells have heralded in a new age in cancer treatment. CAR T therapy has demonstrated remarkable responses in cancer patients of blood cancer [6, 7, 11, 14]. The therapy's success lies in the expression of a chimeric antigen receptor (CAR) that reprograms and redirects T cells to specifically target and attack cancer cells [1]. However, CAR T therapy has faced numerous challenges with attempts to translate this success into solid cancers. Issues related to on-target, off-tumour toxicity [129-132], tonic signalling [104, 146], and T cell dysfunction [147, 149, 150, 151, 155] have limited numerous efforts [18, 21, 108]. However, recent evidence by Eyquem and colleagues demonstrated that CAR expression levels are a critical determinant in T cell activity and therapeutic efficacy –even more so in mediating T cell exhaustion [99]. While insightful, this work assesses only two levels of CAR expression. Thus, there is a notable, outstanding gap in thoroughly understanding how CAR expression levels impact T cell function and response. In this thesis, I report the effects of extensive fine-tuning of CAR expression levels on the T cell transcriptome. First, I adapt microRNA silencing-mediated fine-tuners (miSFITs) technology to tune CAR expression levels in a T cell model. I leverage this model to show that tuning CAR expression correspondingly tunes CAR T cell activation. Next, I translate this technology into primary, human T cells, showing that miSFITs robustly and precisely tune CAR expression. I then demonstrate how tuned CAR expressions controls the expression of CD69 to stepwise levels. Finally, I use single-cell RNA-sequencing to comprehensively dissect the effects of tuning CAR expression in T cell responses, generating a transcriptomic



atlas against step-wise levels of CAR expression. I use this atlas to first demonstrate how tuning CAR expression impacts several genes related to T cell activation, dysfunction, and memory. Using gene signatures, I demonstrate how CAR expression can be optimised to mitigate T cell dysfunction yet spare T cell activation and memory. Embarking on an unbiased discovery analysis, I identify genes, regulons, and signaling pathways that are influenced by CAR expression, providing a novel view on T cell dysfunction. My findings underscore the important role CAR expression levels have on T cell response and provide a promising avenue to address pressing, longstanding challenges hindering the success of CAR-T therapies.

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–A.H.S.R

# List of Abbreviations

In addition to standard abbreviations for metric units (e.g., ml), chemical symbols and formulas (e.g., HCl), amino acids (e.g., three letter code, Ser; or single letter code, S) and nucleic acid organic bases (e.g., G); the following abbreviations and acronyms listed below are used in this thesis.

|                    |                                                    |
|--------------------|----------------------------------------------------|
| .fcs               | Flow cytometry standard file                       |
| 4-1BB              | Tumor necrosis factor ligand superfamily member 9  |
| AF647              | Alexa Fluor 647                                    |
| AICD               | Activation-induced cell death                      |
| ALPPL <sub>2</sub> | Alkaline phosphatase placental-like 2              |
| AP-1               | Activator protein 1                                |
| ATF                | Activating transcription factor                    |
| ATP                | Adenosine triphosphate                             |
| BAM                | binary sequence alignment map format               |
| BATF               | Basic leucine zipper transcription factor ATF-like |
| BCMA               | B-cell maturation antigen                          |
| BH                 | Benjamini-Hochberg                                 |
| BRCA <sub>1</sub>  | Breast cancer type 1 susceptibility protein        |
| BTLA               | B- and T-lymphocyte attenuator                     |

|                    |                                                                                                                                     |
|--------------------|-------------------------------------------------------------------------------------------------------------------------------------|
| BV <sub>421</sub>  | Brilliant Violet 421 dye                                                                                                            |
| bZIP               | Basic leucine zipper transcription factor family                                                                                    |
| C1QBP              | Complement component 1 Q subcomponent-binding protein                                                                               |
| CAR                | Chimeric antigen receptor                                                                                                           |
| CCR <sub>7</sub>   | C-C Chemokine receptor type 7                                                                                                       |
| CD                 | Cluster of differentiation                                                                                                          |
| CD <sub>19</sub>   | Cluster of differentiation 19                                                                                                       |
| CD <sub>3z</sub>   | T-cell receptor T <sub>3</sub> zeta chain                                                                                           |
| cDNA               | Complementary DNA                                                                                                                   |
| CHO-K1             | Chinese Hamster Ovary K-1                                                                                                           |
| CMV                | Cytomegalovirus                                                                                                                     |
| cPPT/CTS           | Central polypurine tract                                                                                                            |
| CRISPR/Cas9        | Clustered Regularly Interspaced Short Palindromic Repeats/Cas9                                                                      |
| CRS                | Cytokine release syndrome                                                                                                           |
| CTLA-4             | Cytotoxic T-lymphocyte-associated antigen 4                                                                                         |
| CXCL <sub>13</sub> | C-X-C motif chemokine ligand 13                                                                                                     |
| DARPs              | Designed ankyrin repeat protein                                                                                                     |
| dCas9              | Nuclease deficient Cas9                                                                                                             |
| DN-28z CAR         | NY-ESO-1 targeting CAR used in this study. Features DN scFv with CD28 costimulatory molecule and CD <sub>3z</sub> signalling domain |
| EC <sub>50</sub>   | Half maximal effective concentration                                                                                                |
| EDTA               | Ethylenediaminetetraacetic acid                                                                                                     |
| EF-1a              | Elongation factor 1a                                                                                                                |
| EGFP               | Enhanced green fluorescent protein                                                                                                  |
| EMax               | Maximum activation                                                                                                                  |
| ETC                | Electron Transport Chain                                                                                                            |
| FACS               | Fluorescence activated cell sorting                                                                                                 |

|          |                                                             |
|----------|-------------------------------------------------------------|
| fastq    | FASTQ file containing biological sequence and quality score |
| FBS      | Fetal bovine serum                                          |
| FDA      | Food and Drug Administration                                |
| FKBP     | FK506 binding protein                                       |
| FLAG     | FLAG epitope: DYKDDDDK                                      |
| FOS      | Fos transcription factor family                             |
| FOSL     | FOS like 1                                                  |
| GATA     | GATA transcription factor family                            |
| gMFI     | Geometric median fluorescence intensity                     |
| GRCh38   | Genome Reference Consortium Human Build 38                  |
| GRN      | Gene regulatory network                                     |
| gRNA     | Guide RNA                                                   |
| HAVCR2   | Hepatitis A virus cellular receptor 2                       |
| HEK293T  | Human embryonic kidney 293 T cell                           |
| HER2     | Human epidermal growth factor receptor 2                    |
| HIV-1    | Human immunodeficiency virus 1                              |
| HLA      | Human Leukocyte antigen                                     |
| HLA-A    | Human Leukocyte antigen A                                   |
| HLA-DPB1 | Major histocompatibility complex, Class II, DP Beta 1       |
| HLH      | Hemaphagocytic lymphohistiocytosis                          |
| HTS      | High throughput sequencing                                  |
| ICANS    | Immune effector cell-associated neurotoxicity syndrome      |
| ICOS     | Inducible T-cell costimulator                               |
| IFN      | Interferon                                                  |
| IgG      | Immunoglobulin G                                            |
| IL       | Interleukin                                                 |
| IL17RB   | Interleukin-17 receptor B                                   |



|                       |                                                                                       |
|-----------------------|---------------------------------------------------------------------------------------|
| IL21R                 | Interleukin-21 receptor                                                               |
| IL7R                  | Interleukin-7 receptor                                                                |
| IRF                   | Interferon regulatory factor transcription factor family                              |
| ITAM                  | Immunoreceptor tyrosine-based activation motif                                        |
| KIR2DS2               | Killer cell immunoglobulin like receptor, two Ig domains and short cytoplasmic tail 2 |
| KNN                   | K-Nearest Neighbor                                                                    |
| KRAB                  | Kruppel-associated box                                                                |
| LAG3                  | Lymphocyte activating 3                                                               |
| LAT                   | Linker for activation of T cells                                                      |
| LAYN                  | Layilin                                                                               |
| LCK                   | Lymphocyte-specific protein tyrosine kinase                                           |
| LTR                   | Long terminal repeat                                                                  |
| MACS                  | Magnetic activated cell sorting                                                       |
| MAFF                  | MAG basic leucine zipper transcription factor F                                       |
| MAS                   | Macrophage activation syndrome                                                        |
| MCAM                  | Melanoma cell adhesion molecule                                                       |
| MHC                   | Major histocompatibility complex                                                      |
| MI                    | mutual information                                                                    |
| miRNA                 | Micro RNA                                                                             |
| miSFITs               | microRNA silencing-mediated fine-tuners                                               |
| MNUD3                 | LTR promoter for U3 region Moloney murine leukemia virus                              |
| MOI                   | Multiplicity of Infection                                                             |
| MRE                   | MicroRNA response element                                                             |
| mRNA                  | Messenger Ribonucleic Acid                                                            |
| MYB                   | MYB proto-oncogene                                                                    |
| nCount <sub>RNA</sub> | Number of counts associated with a cell barcode in a single cell gene expression      |

|                         |                                                                            |
|-------------------------|----------------------------------------------------------------------------|
|                         | matrix                                                                     |
| NAD                     | Nicotinamide Adenine Dinucleotide                                          |
| NFATC                   | Nuclear factor of activated T cells transcription factor family            |
| nFeature <sub>RNA</sub> | Number of genes expressed in single cell gene expression matrix            |
| NFKB1                   | Nuclear factor- $\kappa$ B transcription factor family                     |
| NGFR                    | Nerve growth factor receptor                                               |
| NGS                     | Next generation sequencing                                                 |
| NMI                     | normalised mutual information                                              |
| NR2F6                   | Nuclear receptor subfamily 2 group F member 6                              |
| NR3C1                   | Nuclear receptor subfamily 3 group C member 1                              |
| nt                      | Nucleotide                                                                 |
| NY-ESO-1                | New York esophageal squamous cell carcinoma 1                              |
| ORF                     | Open Reading Frame                                                         |
| OX40                    | Tumor necrosis factor ligand superfamily member 4                          |
| P                       | Probability value                                                          |
| p/ $\rho$               | Pearson's correlation coefficient                                          |
| PBMCs                   | Peripheral blood mononuclear cells                                         |
| PBS                     | Phosphate buffer saline                                                    |
| PCR                     | Polymerase chain reaction                                                  |
| PD-1                    | Programmed cell death protein 1                                            |
| PE                      | Phycoerythrin                                                              |
| PEST                    | Polypeptide sequence rich in Proline, Glutamic Acid, Serine, and Threonine |
| PGK                     | Phosphoglycerate kinase                                                    |
| PRF1                    | Perforin-1                                                                 |
| QC                      | Quality Control                                                            |
| R <sup>2</sup>          | R squared value                                                            |
| RISC                    | RNA induced silencing complex                                              |

|                                |                                                         |
|--------------------------------|---------------------------------------------------------|
| RNA                            | Ribonucleic acid                                        |
| RNA-seq                        | RNA sequencing                                          |
| RNU6b                          | RNA U6 small nuclear 6                                  |
| RRE                            | Rev response element                                    |
| RUNX                           | Runx transcription factor family                        |
| RXRA                           | Retinoid X receptor alpha                               |
| S <sub>1</sub> PR <sub>1</sub> | Sphingosine-1-phosphate receptor 1                      |
| SCENIC                         | Single-Cell rEgulatory Network Inference and Clustering |
| scFv                           | Single-chain variable fragment                          |
| scRNA-seq                      | Single cell RNA sequencing                              |
| SELL                           | L-selectin                                              |
| sgRNA                          | Single guide RNA                                        |
| TCR                            | T-cell receptor                                         |
| T <sub>CM</sub>                | Central memory T cell                                   |
| T <sub>EFF</sub>               | Effector T cell                                         |
| T <sub>EXH</sub>               | Exhausted T cell                                        |
| T <sub>FH</sub>                | Follicular helper T cell                                |
| TGFB <sub>1</sub>              | Transforming growth factor-beta                         |
| T <sub>H</sub>                 | Helper T cell                                           |
| TF                             | Transcription factor                                    |
| TIGIT                          | T cell immunoreceptor with Ig and ITIM domains          |
| TIM <sub>3</sub>               | T-cell immunoglobulin and mucin domain 3                |
| T <sub>M</sub>                 | Memory T cell                                           |
| T <sub>N</sub>                 | Naive T cell                                            |
| TNF                            | Tumor necrosis factor                                   |
| TOX                            | Thymocyte selection-associated HMG box                  |
| TRAC                           | T-cell receptor alpha locus                             |

|                     |                                                                        |
|---------------------|------------------------------------------------------------------------|
| $T_{REG}$           | Regulatory T cell                                                      |
| $T_{RM}$            | Resident memory T cell                                                 |
| TRUCKs              | T cells redirected for antigen-unrestricted cytokine-initiated killing |
| $T_{TERM}$          | Terminally exhausted T cell                                            |
| Ub                  | Ubiquitin                                                              |
| UCP <sub>2</sub>    | Mitochondrial uncoupling protein 2                                     |
| UFD                 | Ub-fusion degradation                                                  |
| UMAP                | Uniform Manifold Approximation and Projection for dimension reduction  |
| uORFs               | upstream Open Reading Frame                                            |
| UMI                 | Unique Molecular Identifier                                            |
| UTR                 | Untranslated Region                                                    |
| V <sub>450</sub>    | BD Horizon V <sub>450</sub> fluorephore                                |
| $V_H$               | Variable heavy chain                                                   |
| $V_L$               | Variable light chain                                                   |
| VP <sub>64</sub>    | 4 tandem copies of Herpes Simplex Viral Protein 16                     |
| WPRE                | Woodchuck hepatitis virus post-transcriptional regulatory element      |
| WRNIP <sub>1</sub>  | WRN Helicase interacting protein 1                                     |
| ZAP-70              | Zeta-chain-associated protein kinase 70                                |
| ZFP <sub>36</sub>   | Tristetraprolin                                                        |
| ZFP <sub>36L2</sub> | ZFP <sub>36</sub> Ring Finger Protein Like 2                           |

*DECLARATION OF AUTHORSHIP*

THIS THESIS, AND THE WORK PRESENTED HEREIN IS MY OWN UNLESS OTHERWISE SPECIFIED. THIS WORK WAS CARRIED OUT PRIMARILY AT THE WEATHERALL INSTITUTE OF MOLECULAR MEDICINE, UNIVERSITY OF OXFORD. I AM SUBMITTING THIS THESIS FOR THE DEGREE OF DPHIL IN MEDICAL SCIENCES. THIS THESIS HAS NOT BEEN SUBMITTED FOR ANY OTHER DEGREE.

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# 1

## Introduction

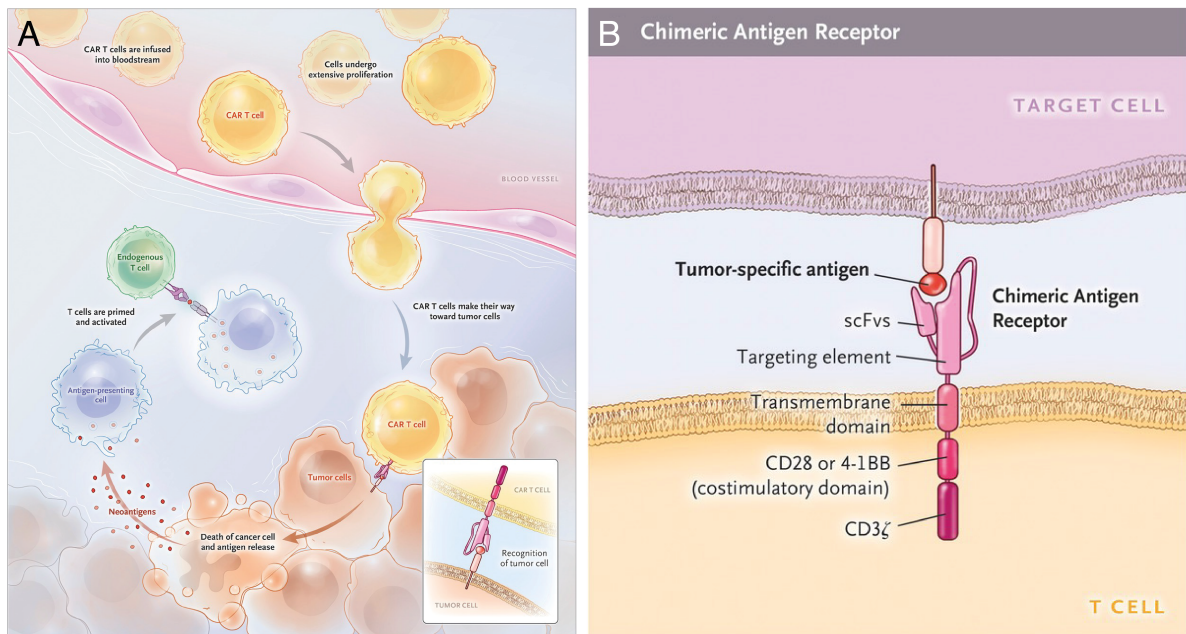
IN THIS THESIS, I DOCUMENT MY EFFORTS to dissect the impact of tuning chimeric antigen receptor (CAR) expression in the novel, cell-based immunotherapy therapy, CAR-T cells. This introductory chapter begins by discussing what CARs are and their success as a therapy. Next, I consider and highlight key limitations and challenges of the therapy. In doing so, I justify why tuning CAR expression is an avenue worth exploring and a key parameter worth characterizing. I then discuss gene tuning strategies employed in mammalian cells before highlighting a robust, novel technology recently developed that uses engineered miRNA response elements. This introduction ends with how I propose to harness this technology to tune CAR expression levels and subsequently dissect T cell response.

## 1.1 CHIMERIC ANTIGEN RECEPTOR T CELL THERAPY

Chimeric antigen receptor (CAR) T cell therapies have emerged as the poster child of success in the field of cancer immunotherapy due to their resounding success in the treatment of blood cancers [1, 2]. Indeed, durable clinical responses and complete remission have been observed in a significant proportion of patient populations for acute lymphoblastic leukaemia, chronic lymphocytic leukaemia, non-Hodgkin lymphoma, follicular lymphoma, mantle cell lymphoma, and, more recently, multiple myeloma [3-17]. For example, in one of the first clinical trials, 90% of patients achieved complete remission [14]. This has led multiple regulatory bodies such as the European Medicines Agency, the NHS's National Institute for Health and Care Excellence, and U.S. Food and Drug Administration to grant approval for such therapies, heralding a new era in cancer treatment.

Chimeric antigen receptor T cell therapy functions through the expression of a chimeric antigen receptor (CAR) in a patient's T cells. CARs are synthetic receptors that redirect the specificity and function of T cells to target and elicit cytotoxicity against cancer cells (Figure 1.1a). CARs are modular proteins and consist of four main components: 1) the target-specifying extracellular domain, 2) the flexible hinge domain, 3) the anchoring transmembrane domain, and 4) the intracellular signalling domain (Figure 1.1b) [18-24]. Each domain has notable design considerations that influence CAR T cell function. Another design aspect related to CAR T cell therapy is the manufacturing process, or how the CAR is introduced into the T cell. Below, I will discuss each aspect's role within CARs with the goal of providing a basis for how each component contributes to CAR T functionality, behaviour, and, notably, eventual limitations.

The extracellular domain endows antigen recognition and binding to CAR-Ts. The extracellular domain is most often a single-chain variable fragment (scFv), which is comprised of variable heavy ( $V_H$ ) and light ( $V_L$ ) immunoglobulin chains, derived from monoclonal antibodies[25-28]. These  $V_H$  and  $V_L$  chains confer specificity by their recognition sites, allowing them to bind to specific epitopes present on target cells[25-28].  $V_H$  and  $V_L$  chains are connected by a flexible linker, often composed of Glycine and Serine peptides, incorporated to provide solubility and flexibility in folding and orien-



**Figure 1.1: Chimeric Antigen Receptor (CAR) T cell therapy and CAR structure.** A) Once infused into a patient, CAR-T cells traffic, bind to, and elicit cytotoxicity against cancer cells. B) The structure of a general chimeric antigen receptor. Reproduced with permission from June, C.H. and M. Sadelain, Chimeric Antigen Receptor Therapy. New England Journal of Medicine, 2018. Copyright Massachusetts Medical Society [1].

tation [29].

Classically, scFvs target extracellular antigens of cell-surface proteins expressed on cancer cells. For example, current FDA-approved CAR-T therapies targets include B cell associated markers CD19 and BCMA, while clinical trials have demonstrated promising results with CARs targeting CD20 and CD22. A notable advantage of scFv-based CARs over the endogenous T cell receptor (TCR) is that they proffer major-histocompatibility complex (MHC)-independent target recognition. However, efforts have led to the development of T cell receptor-like CARs that recognise MHC-presented antigens [30-32].

Interest to expand the CAR target repertoire has led to numerous studies to develop novel CARs featuring engineered scFvs. For example, work by Chang and colleagues [33] demonstrated dimerizing ligands can activate CARs by binding them in *cis*. Chang and colleagues highlighted this concept in CARs targeting TGF- $\beta$ , thus harnessing this immunosuppressive molecule into a potent activator in

CAR-T therapy.

Further work has expanded CAR extracellular domains to completely alternative antigen-binding domains. For example, Xie and colleagues used nanobodies in CARs targeting EIIIB, a splice variant of fibronectin overexpressed in the tumor microenvironment. Nanobodies are small, single-domain, heavy-chain only antibody fragments that have similar affinities to scFv. Due to their size, nanobodies can target different epitopes inaccessible to scFvs. In their studies, Xie et al demonstrated tumour growth reduction that was comparable to equivalent to CARs targeting the same antigen but using an scFv extracellular domain [34, 35].

Exciting work demonstrated CARs using tethered cytokines to target their respective receptors. For example, Brown and colleagues demonstrated a zetakine-CAR featuring a membrane-tethered IL-13 extracellular domain, thus specifying the CAR for IL-13 receptor  $\alpha 2$ . They harnessed this CAR in numerous clinical studies, portraying effective tumour growth reduction in intracranial and spinal glioblastomas and numerous patients [36-38].

Designed ankyrin repeat proteins (DARPs) have also been harnessed as extracellular domains in CARs. DARPs are small, antibody-mimetic proteins of high thermodynamic stability [39], consisting of one polypeptide chain that forms a functional binding domain, and reduced clustering compared to scFvs. Hammill and colleagues demonstrated cytokine secretion and cytotoxicity using anti-HER2 DARP CARs [40].

These different extracellular domains have often been guided by interest to expand the target repertoire of CARs. Indeed, ongoing CAR-T clinical trials and research efforts have targeted a wide variety of tumour-associated and specific antigens, providing safe, targets that spare any healthy cells from damage. Currently investigated targets are tabulated in the Appendix [1, 24, 41-44].

There are other key considerations related to the extracellular binding domain outside of recognition and binding. A key component relates to the affinity of a CAR for its specified target. For example, scFv affinity is a fundamental determinant for functionality and needs to be high enough to recognise cancer cells and induce CAR signalling and T cell activation. However, studies have demonstrated excessively high affinity can give rise to activation-induced cell death (AICD) and toxic-

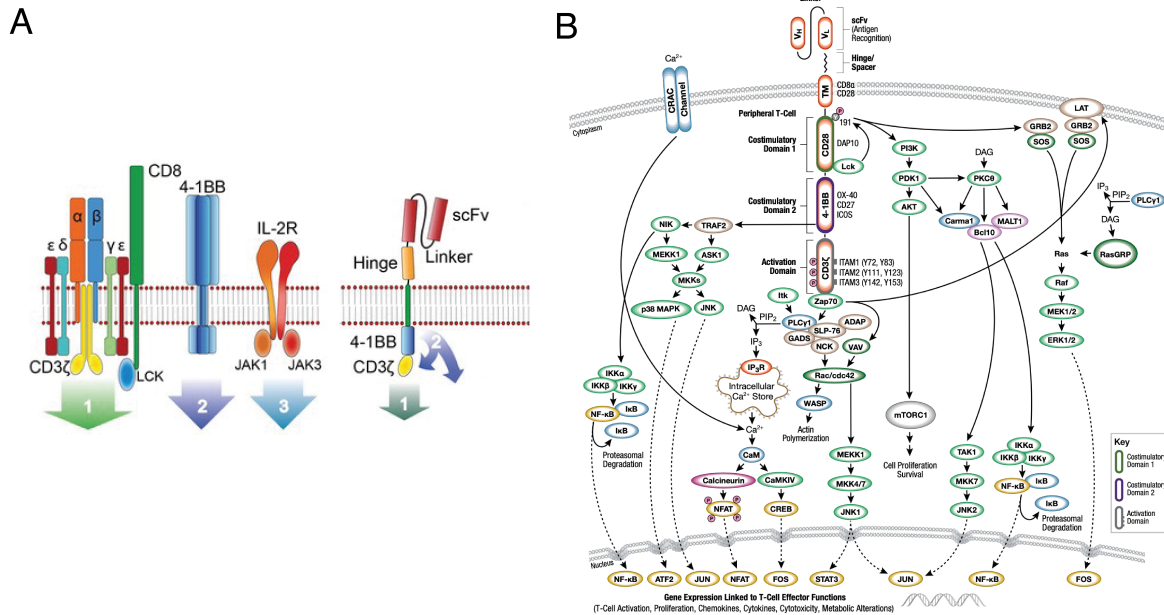
ities [45-49]. Furthermore, some extracellular domains, most notably scFvs, have been demonstrated to spontaneously cluster, giving rise to ligand-independent tonic signalling, discussed in depth later in this chapter [50, 51].

Thus, the extracellular domain of CARs is a key component of CAR-T functionality, proffering specificity for numerous targets and cancers but also eliciting design considerations for safety.

Immediately downstream of the extracellular domain is the hinge domain, also known as the spacer. The hinge domain separates the CAR's extracellular binding units from its transmembrane domain. Further, it provides stability for efficient CAR expression and activity [19]. This domain provides flexibility to overcome steric hindrances and to provide adequate length for access to target antigens by the extracellular domain [19-21, 23, 52-54]. Indeed, studies have demonstrated that optimal spacer length is motivated by the position of the targeted epitope. For example, longer spacers allow better access to membrane-proximal epitopes [52], while shorter spacers more efficiently bind membrane-distal epitopes [46, 55]. Common hinge sequences used are those derived from native T-cell molecules CD8 and CD28 or from immunoglobulins (IgG), such as CH<sub>2</sub> and CH<sub>3</sub> [19, 55]. However, IgG-derived hinges have been demonstrated to retain their endogenous ability to bind to Fc gamma receptor (FcγR), eliciting off-target activation and cytotoxicity. Majzner and colleagues demonstrated increased functionality using a CAR with a CD28 hinge domain compared to those with a CD8 hinge. In their studies, the CD28 domain CARs demonstrated faster killing times post immune synapse engagement, conferring superior tumour control and survival *in vivo* [54]. Overall, the hinge domain influences the extracellular antigen sensing domain's ability to bind targets, providing a notable design consideration in CAR-Ts.

The transmembrane domain anchors the CAR in the T cell membrane. Most CARs to date have been created with single-spanning transmembrane domains derived from CD3ζ, CD4, CD8a, or CD28. [56-58]. The domain used has been shown to influence the stability and function of the CAR [59, 60]. For example, CARs containing CD28 transmembrane domains were more stable than CD3ζ transmembrane domain containing CARs [61]. Furthermore, CD8 transmembrane domain CARs secreted less IFNγ and TNF but are less susceptible to activation-induced cell death (AICD) [62].





**Figure 1.2: Overview of CAR signalling.** A) Activation signals within the T cell receptor (left) and within a CAR (right). B) Signalling cascades involved in CAR T cell activation. Signals propagate to eventually to activate transcription factors, such as NFAT (nuclear factor for activated T cells) and NF- $\kappa$ B (nuclear factor kappa Beta). Subfigure A adapted under Creative Commons NonCommercial License (CC BY-NC 4.0) from Lindner, S.E., et al., Chimeric antigen receptor signaling: Functional consequences and design implications. Science Advances, 2020. [23]. Subfigure B illustration reproduced and adapted courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).

In essence, the transmembrane domain represents a relatively unexplored yet essential component in CAR design.

The final domain of CARs is the endodomain, or the intracellular signalling domain (Figure 1.2), and is the core mechanism of function for CAR-Ts. It is typically comprised of an activation domain and one or more co-stimulatory domains. Most CARs feature an activating domain derived from CD3 $\zeta$  immunoreceptor tyrosine-based activation motifs (ITAMs), the signalling domains of the T cell receptor  $\zeta$  chain. However, signalling mediated solely by this signalling domain is insufficient to fully activate T cells and has demonstrated limited T cell persistence and activity in vivo [63]. Thus, a co-stimulatory signal is required for optimal function, metabolism, and persistence [64].

The most widely used co-stimulatory domains are derived from CD28 or 4-1BB (CD137) and are featured in all FDA-approved CAR-T therapies [65]. Notable differences have been observed when comparing CD28- and 4-1BB-containing CAR functionality and metabolism [66-71]. For example,

CD28 CAR Ts have been shown to elicit more rapid T cell activation, proliferation, and cytotoxicity; predominantly differentiate into effector memory T cells; and utilise aerobic glycolysis. In contrast, 4-1BB CAR Ts induce a slower T cell effector response; have a higher and more sustained persistence; predominantly differentiate into central memory T cells; and have increased mitochondrial biogenesis and oxidative metabolism [21, 60, 67, 72, 73]. While the majority of CARs feature CD28 or 4-1BB costimulatory molecules, other co-stimulatory molecules, such as CD40, OX40, ICOS, CD27, and KIR2DS2, have been used instead and produced promising CAR T cell performance [74-80].

Substantive research has been conducted on the CAR endodomain, leading to the development of multiple CAR generations, as commonly termed. This iterative development has progressively recapitulated the following canonical T cell receptor activation events (Figure 1.2) [23]:

- Signal 1: T cell receptor-mediated signalling elicited after antigen binding. This signal is led by lymphocyte-specific protein tyrosine kinase (LCK) for the phosphorylation of the TCR complex's ITAMs (Figure 1.2b). This ultimately leads to the recruitment of Src homology containing kinases, such as zeta-chain-associated protein kinase 70 (ZAP-70), which further activate downstream signalling proteins, such as linker of activated T cells (LAT), which eventually lead to the activity of transcription factors (Figure 1.2b) [81-84]
- Signal 2: Costimulation by CD28, 4-1BB, and OX40 T cell surface molecules, preventing T cell anergy and achieving optimal activation through assisting in the formation of the immunological synapse [85, 86]
- Signal 3: Cytokine signalling, mediated by molecules such as IL-2, IL-7, and IL-15, occurring after signals 1 and 2 but required for optimal T cell activation [86-88].

As discussed, first-generation CARs are composed solely of a CD3ζ signalling domain's 3 ITAMs (Figure 1.2b), providing the needed phosphorylation sites post-ligand binding. Thus, the first-generation CAR design only incorporates signal 1 of T cell activation. These CARs ultimately fail in activating and inducing persistent T cells, likely being explained by the lack of costimulation, signal 2, leading

to anergy and inefficient activation. As described, this issue was overcome by coupling the CAR's CD3 $\zeta$  signalling domain with a costimulatory molecule, providing the second signal and giving rise to second-generation CARs (Figure 1.2A).

Further generations incorporate numerous additions. In third-generation CARs, both CD28 and 4-1BB co-stimulatory domains are included, proffering enhanced cytokine secretion, *in vivo* T cell survival and tumour elimination [71, 73, 89, 90]. In fourth and fifth-generation CARs, second-generation CAR architectures (CD3 $\zeta$  signalling domain and a costimulatory molecule) are paired with further additions. In 4th generation CARs, known as T-cell redirected for universal cytokine-mediated killing (TRUCKs), an induction domain for the expression of a chemokine, such as IL-12 or IL-18, is included [91-93]. This has provided these CAR-Ts with stimulating cytokines that enhance expansion and persistence despite the immunosuppressive tumour microenvironment.

Fifth-generation CARs endeavour to fully recapitulate all three signals of canonical TCR-mediated activation [23]. In these CARs, the cytoplasmic tail of the IL-2 receptor  $\beta$ -chain cytokine receptor (IL-2R $\beta$ ) with a binding site for the STAT3 transcription factor was incorporated alongside the CD3 $\zeta$  signalling domain and costimulatory molecule in the endodomain [94]. CAR-Ts featuring this architecture demonstrated robust proliferation and reduced terminal differentiation *in vitro*. *In vivo* these CARs demonstrated superior antitumor effector function and persistence to second-generation CARs [94].

Another aspect of CAR T therapy is the method to introduce and express CARs in T cells. Standard manufacturing protocols for both pre-clinical studies and FDA-approved therapies utilize bulk retroviral transduction techniques, mediated by gamma retrovirus and lentivirus. This ultimately gives rise to the CAR transgene stably integrating within the genome, allowing for long-term expression. However, retrovirus and lentivirus integrate in a semi-random nature in the host genome, posing the risk for insertional mutagenesis and dysregulation of genes adjacent to integration site [95-98]. Alternative methods to introduce the CAR transgene into T cells have been applied and demonstrated robust integration and stable expression, as seen in CRISPR/Cas9 and the Sleeping Beauty transposon system [99, 100].

As CARs are introduced into cells by retrovirus or lentivirus, they are housed in lentiviral or retroviral backbone vectors. In the case of retrovirus, CAR expression would be driven by the retroviral LTR enhancer and promoter elements. In lentiviral vectors, user-decided promoters drive expression. In many cases, this is a strong, constitutive promoter, such as the phosphoglycerate kinase (PGK), cytomegalovirus (CMV), LTR promoter for U<sub>3</sub> region Moloney murine leukaemia virus (MNUD<sub>3</sub>) [101, 102], or elongation factor 1 $\alpha$  (EF-1 $\alpha$ ) [97, 103-4]. Another related factor to CAR vector expression lies in the number of vector copies integrated into the genome, known as the insertion frequency. This ultimately influences the level of transgene expression. The multiplicity of infection (MOI) used during the transduction strongly dictates the insertion frequency. These factors all converge to determine the overall, resulting CAR expression level in the T cell.

Overall, several parameters and decisions go into CAR T design, giving rise to a complex, nuanced, and smart therapy to combat cancer. However, despite this, there are still notable limitations within CAR T therapy.

## 1.2 CHALLENGES OF CAR-THERAPY

Despite their resounding success, CAR-T therapy has encountered numerous challenges. In this section, I will consider some of the most pressing and relevant limitations preventing the further success and wider dissemination of CAR-T therapies.

In clinical studies, the majority if not all patients encounter severe, toxic side effects, many of which are associated with on-target, on-tumor effects [18, 105, 106]. These occur when there is a direct attack by CAR-Ts on normal, healthy cells that have shared expression of the targeted antigen. These include immune effector cell-associated neurotoxicity syndrome (ICANS), hemaphagocytic lymphohistiocytosis (HLH) and macrophage activation syndrome (MAS), and cytokine release syndrome (CRS) [1, 18, 19, 42, 107-111].

ICANS, often understood as neurotoxicity, often manifests as toxic encephalopathy. Common symptoms include reduced attention, confusion, reduced motor capacity, seizures, comas, elevated

intracranial pressure, and cerebral edema. ICANS has been recorded to affect from up to 91% of patients receiving CAR-T therapy [109, 111-114].

HLH and MAS are both characterised by excessive inflammation and abnormal proliferation of lymphocytes and macrophages, resulting in the excessive presence and secretion of inflammatory cytokines. Core manifestations of both include fevers, swelling of the liver and spleen, abnormal liver function, and decreased blood cells. It is a rare disease, occurring in less than 4% of patients, but has a significantly high mortality (up to 80% death) [110, 111, 114-116].

CRS, also known as “cytokine storms,” is a systemic inflammatory response associated with T cell activation and the rapid and excessive secretion of inflammatory cytokines, which further induce other T cells to secrete cytokines. It is characterised by fevers, tachycardia, hypotension, hypoxia, and capillary leakage. CRS occurs commonly. Clinical studies have recorded 50-93% of patients having varying levels of CRS severity in CAR-T therapy [109, 111, 117-120].

The root cause of these toxic-side effects has not been fully established, but all have similarly been attributed to excessive levels of IL-1, IL-2, IL-6, IL-10, IL-15, TNF- $\alpha$ , and IFN- $\gamma$  [121-123]. When activating, CAR-Ts have been demonstrated to release these cytokines constitutively and at excessive levels [104, 111, 124, 125]. This has been shown to recruit other immune cells (e.g. other T cells, macrophages, and monocytes) into activating and producing further inflammatory cytokines. This further recruits and activates other immune cells, forming a positive feedback loop of inflammation [111]. This is likely further compounded by the fact that CAR-Ts elicit prolonged exponential expansion [104]. Numerous control mechanisms have been developed with the goal to balance CAR-T activation and effector function with the toxic side effects resulting from unregulated overaction [126]. However, despite these attempts, toxicity is still primarily managed through the dosage of inflammatory inhibitors, such as tocilizumab, or glucocorticoids, such as dexamethasone [1, 18, 44, 108, 127]. Thus, this is an outstanding issue that hinders CAR-T therapy. However, given the role of activation in these toxicities, this issue spotlights a gap in knowledge in T cell activation and posits a critical question: could T cell activation be correspondingly tuned to be contained and regulated?

The successes in CAR-T therapy are limited to blood cancers. CAR-T therapy has encountered

significant challenges that have precluded their translation into solid tumour cancers. These include tonic signalling, T cell dysfunction, and on-target, off-tumour toxicity.

Another type of CAR-T toxicity relates to on-target, off-tumour toxicity, also understood as lack of specificity. Indeed, even in the successful Kymriah and Yescarta therapies, CAR-Ts target CD19, which has broad and high expression in healthy B-cells in addition to B-cell leukaemia's and lymphomas[1]. Thus, B-cell aplasia is an observed yet predicted on-target, off-tumour adverse effect. However, it is managed by immunoglobulin G (IgG) supplementation [128].

Many of CAR-T targets in solid tumours are not tumour-specific. Indeed, they are often tumour-associated antigens that are expressed on healthy tissues at varying levels. Depending on antigen's expression pattern on human tissues, management plans to address any healthy cell killing may not be possible, as is done in CD19 targeting CARs. Further, off-tumour targeting can be dangerous [129-131] and lethal, as observed in a patient receiving CAR-Ts targeting HER-2, a tumour-associated antigen expressed on numerous cancers. In this instance, a patient experienced respiratory distress 15 minutes post CAR-T cell infusion and ultimately perished 5 days later [132]. Clinicians overseeing the administration speculated that CAR-Ts immediately localised to the lungs post-infusion and were triggered to release cytokine in response to low levels of HER-2 on lung epithelial cells. This highlights the gravity of off-tumour targeting the severe need for solutions.

The majority of efforts to address this issue have centred on the CAR extracellular domain. For example, AND-logic gated CAR-Ts have been developed [20, 125, 133-138]. A notable deployment of this strategy features synNotch CAR Ts circuits. Hyrenius-Wittsten and colleagues generated T cells expressing synthetic Notch receptors that sensed mesothelioma-specific antigen ALPPL2, alkaline phosphatase placental-like 2 [139]. When primed, the cleavage of the Notch intracellular was induced, driving the transcription of CARs specific for melanoma cell adhesion molecule (MCAM)-expressing tumours. Excitingly, this system displayed robust anti-tumour potency and antigen specificity [139].

Furthermore, numerous efforts using affinity tuning of the CAR's scFv to reduce off-tumour toxicity have emerged [48, 140]. Work by Liu and colleagues developed a panel of CARs using scFvs fea-

tuning point mutations throughout the heavy and light chains [48]. These point mutations changed the affinity of the scFv and, therefore, the CAR. Liu and colleagues harnessed these tuned-affinity CARs and demonstrated robust tumour killing yet reduced toxicity to healthy tissue [48].

Tuned CAR expression represents a unique opportunity to mitigate on-target, off-tumour toxicity. For example, as observed in Liu and colleagues' work, CAR expression could be similarly tuned to an ideal level that optimally maintains specificity against tumour cells and reduces off-targeting to healthy, normal tissue. Taken another way, tuning CAR expression levels could be an avenue to endow CAR-T's with the specificity to differentiate between cells of different antigen densities.

Tonic signalling is the non-coordinated and sustained activation of a CAR T-cell in a ligand-independent or dependent fashion [50, 60, 99, 141, 142]. In ligand-dependent tonic signalling, target antigens of CARs persist, as observed in cancer and solid tumour microenvironment. Thus, the CAR molecules on the T cell surface are constantly interacting and binding with antigens, thus chronically triggering and sending CAR activation signals. In ligand-independent signalling, CARs spontaneously aggregate and cluster on the T cell surface, giving rise to physical interactions between the separate CAR molecules. This ultimately triggers the activation of CAR T cells independent of target antigen binding [104, 141-144].

In both cases, tonic signalling has been long associated with impaired T cell effector function and survival. Tonic signalling can give rise to T cell dysfunction or exhaustion [142, 143]. Indeed, tonic signalling is associated with chronic cell activation [45, 70, 104, 143, 145], characterised by constitutive phosphorylation of the CD3ζ intracellular domain [19, 50, 141, 143]. Further, it has been attributed with the constitutive secretion of excessive amounts of cytokines [104, 146], a behaviour also seen in toxicity [121, 123, 126], as discussed previously. Finally, tonically signalling CAR-Ts demonstrate accelerated T cell differentiation and exhaustion [147-149].

There have been several suggested causes for tonic signalling, many of which are attributed to CAR design. For example, scFvs that are prone to aggregation have been suggested to drive tonic signalling at lower CAR densities [45, 50, 104, 146]. Further, extracellular domain and hinge combinations are often selected to confer increased flexibility and navigate steric hindrances. However, this likely comes

at the significant cost of spontaneous binding and activation [45, 70, 104, 146].

One commonly suggested, yet sparsely explored approach entails controlling the level of CAR expression. In ligand-independent tonic signalling, reduced levels of CAR expression would reduce the random, physical interactions between other CAR molecules that would send an activation signal [45, 104]. In ligand-dependent signalling, reduced CAR expression could reduce the likelihood of interaction with antigen. Thus, tonic signalling represents a significant challenge that could be well-addressed by tuning CAR expression.

T cell exhaustion, a form of dysfunction, is a cell state that has hamstrung many immunotherapies but has especially impacted CAR-T therapies. T cell exhaustion is a physiological, hypoactive T cell state that is marked by a progressive loss of key T cell functionality [147, 150-154]. This includes reduced proliferation [155-157] and persistence capability [156, 158, 159] and diminished secretion of key effector molecules [155, 159-163], such as cytokines, perforins, and granzymes [148, 149, 152, 164-167]. It has been associated with increased chemokine expression, increased excessive and terminal differentiation [168-171], and the upregulation of multiple inhibitory receptors [172-174], such as PD1, TIM3, LAG3, CTLA4, and TIGIT [148, 149, 153, 154, 158, 166, 167]. Furthermore, studies have revealed an altered transcriptional programme and epigenetic landscape [111, 148, 163, 164, 168, 175-178].

It is well understood that the exhaustion state is induced as a result of chronic stimulation of the CAR [50, 99, 141, 148, 149, 154, 164, 176, 178-181]. In the case of solid cancers, CAR Ts infiltrate into the tumour microenvironment and are faced with high levels of target antigen. This leads to multiple binding events, giving rise to ligand-mediated chronic signalling. Furthermore, CARs are optimised for efficient binding to antigens and for receiving strong TCR- and co-stimulation. Thus, when faced with numerous binding events, the CAR T elicits excessively strong signals, adding further fuel to exhaustion. On top of this, external, inhibitory factors present in the tumour microenvironment, such as hypoxia and immunosuppressive cytokines IL-10 and TGF- $\beta$ , serve as compounding factors, further driving the exhaustion process [147, 150, 151, 154, 177, 178, 182].

This state of dysfunction ultimately results in CAR T therapies that are ineffective and fail to pro-



duce significant responses in patients [95, 183]. Numerous strategies have been devised to combat T cell exhaustion [95, 126, 176, 180, 183].

One strategy described by Lynn and colleagues involved the overexpression of c-Jun [180]. In studying transcriptional and epigenetic programmes associated with tonic signalling CARs, Lynn and colleagues identified the AP-1 transcription factor family being enriched in naïve CAR-expressing T cells. The AP-1 transcription factor family form homo- and heterodimers through interactions in the bZIP domain and can complex with IRF transcription factors. When measuring the relative activity of different transcription factors in the AP-1 family, they observed reduced expression of c-Jun. c-Jun is a canonical activator protein-1 (AP-1) transcription factor with roles in numerous cell activities, such as proliferation, apoptosis, and survival [184]. It heterodimerizes with c-Fos to drive *IL2* expression. Importantly, other transcription factor complexes comprised of AP-1 and IRF transcription factors have been shown to antagonize c-Jun activity and drive immunoregulatory gene expression in T cells [185-188]. Given the lower level of c-Jun activity, Lynn and colleagues hypothesized that dysfunction in exhausted T cells was related to the deficiency in c-Jun-c-Fos AP-1 heterodimers. When introducing an expression vector that overexpressed c-Jun alongside their CAR, they observed an increased frequency of stem-cell-memory and central-memory T cell subsets in exhausted CAR T cells.

Lynn and colleagues harnessed c-Jun overexpression and demonstrated exhaustion-resistant CAR-Ts. These CAR-Ts were marked with improved anti-tumour potency, diminished terminal differentiation, and enhanced expansion potential both *in vitro* and *in vivo*. Lynn and colleagues' work demonstrates how the supplemental expression of a key factor in the T cell transcriptome can be harnessed to combat exhaustion.

Weber and colleagues developed an alternative approach involving 'transient rest' that led to the reversal of T cell exhaustion [176]. In one instance of their approach, they used FK506 binding protein 12 (FKBP) degradation domain (DD). FKBP is a mutated protein that serves as a degradation domain when tagged to a protein [189]. Its degradation capacity is counteracted by shield-1, which serves to stabilise the protein FKBP is tagged to. Thus, in this system, the CAR is degraded by the proteasome in the absence of shield-1. However, when shield-1 is introduced, the CAR is stabilised and able to

elicit its functionality. Weber and colleagues leveraged this system, degrading the CAR at various time points and for various lengths, introducing ‘rest’ periods. Importantly, this leads to the cessation of antigen-induced CAR signalling. Weber and colleagues developed an additional, similar transient inhibition of CAR using dasatinib. Dasatinib interrupts tyrosine kinase pathways of CAR signaling, thus suppressing T cell activity. Weber and colleagues introduced dasatinib to induce periods of rest in the CAR but removed it to restore CAR function.

When subjecting tonically signalling, pre-exhausted CAR T cells to any period of rest, Weber and colleagues observed reinvigorated, anti-tumour functionality. For example, rested CAR T cells were able to once again secrete key cytokines IL-2, IFN $\gamma$ , and TNF $\alpha$ , and enhanced tumour growth control compared to unrested controls. Furthermore, they demonstrate the enrichment of memory-associated genes and memory-associated transcription factor motifs and the reduction of exhaustion genes in rested CAR-Ts. This was further replicated in proteomic analysis. Rested CAR T cells demonstrated a lower exhaustion score (mean expression of PD-1, TIM-3, LAG-3, CTLA-4, BTLA, 2B4, and CD39) but a higher memory score (mean expression of CD45RA, IL-7R, CD27, and CD197). Taken altogether, transient rest a unique strategy to rescue CAR-Ts from exhaustion.

These two strategies represent promising avenues to tackle T cell exhaustion; however, there are notable limitations with both. Lynn’s c-Jun method requires the additional overexpression of c-Jun, which has a suggested oncogenic role [190, 191]. Thus, c-Jun overexpression can enhance the risk of other toxicities. Weber’s transient rest method requires the application of an exogenous component, potentially introducing confounding effects on T cell function or impacting other cells. Even more, dasatinib interrupts tyrosine kinase pathways of CAR signaling. Its application would have to incorporate into an ON/OFF dosing scheduling, strategically during periods of tumour growth or waves of inflammatory cytokines. In these instances, reinvigoration of T cell immunity could elicit lethal hyperinflammation [192]. As such, much more work is required before these strategies wholly address T cell exhaustion.

The challenge of exhaustion has not been fully resolved. Another potential strategy to mitigate exhaustion is to control the expression level of the CAR transgene. Indeed, seminal work conducted

by Eyquem and colleagues inadvertently provided clear evidence that optimising CAR expression levels can be a possible solution to these outstanding issues. In this study, Eyquem and colleagues introduced the CAR transgene into the endogenous T cell receptor alpha chain (*TRAC*) locus using CRISPR/Cas9. This disrupted the expression of the T cell receptor alpha chain, effectively knocking out the expression of the TCR. In its place was the CAR transgene, placed under the expression and regulation of the endogenous *TRAC* promoter.

Knockout of the endogenous T cell receptor (TCR-KO) provides the notable advantage of mediating a challenge in CAR-T manufacturing: graft versus host disease (GvHD) [401-3]. GvHD occurs when infused, donor T cells view the recipient's cells as foreign and therefore launch an immune response [404-406]. Indeed, recent work has demonstrated TCR-KO CAR-Ts to elicit robust anti-tumour efficacy both in vitro and in vivo, comparable to CAR-T of standard manufacturing, while demonstrating an ablation of alloreactivity [401-3]. Thus, the knockout of the endogenous TCR provides the benefit of expanding the sources of T cells for CAR-T manufacturing. Similarly, controlling TCR expression levels would also be of interest. However, no work reported thus far have been able to successfully do so. While purely speculative, controlling TCR expression could be used to answer fundamental, biological questions such as expression levels' role in T cell development and subtype specification, TCR sensitivity and specificity, and receptor signalling. These would provide valuable insights to outstanding questions that remain due to a lack of robust tuning technologies.

This engineering resulted in T cells with reduced and uniform CAR expression compared to virally transduced controls. Remarkably, these cells exhibited enhanced T cell potency and survival rates *in vivo* compared to CAR-Ts produced using retroviral transduction, the standard manufacturing practice. Even more, these cells demonstrated reduced constitutive signalling and T cell exhaustion and delayed terminal differentiation. Interestingly, they observed CAR downregulation and internalisation, akin to the natural regulation of the endogenous T cell receptor [366]. Simply put, this study indicates that CAR expression level is a critical determinant of T cell activity and therapeutic efficacy.

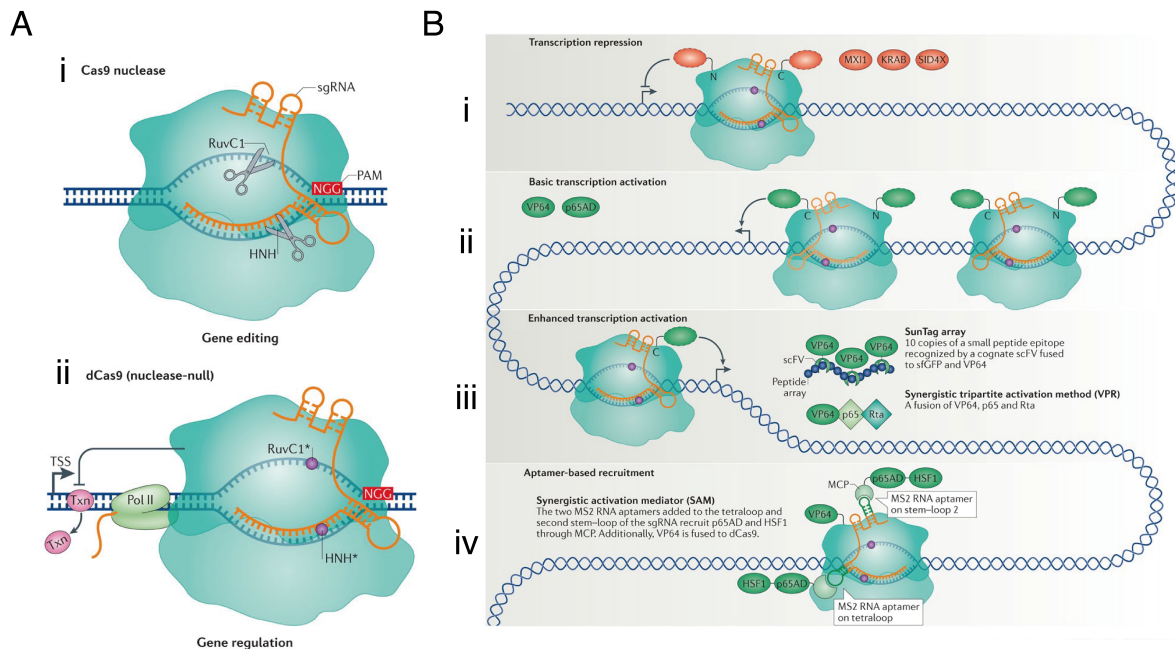
However, this study effectively only compared two levels of expression, and while the results were noteworthy, they were limited. I sought to expand upon Eyquem and colleagues' findings by more

comprehensively characterising the relationship between CAR expression levels and T cell state. Specifically, I wanted to drastically increase the number of expression levels studied and characterise the impact of CAR expression levels on the T cell response in a comprehensive manner. Given Eyquem et al's findings, this knowledge could be harnessed to address the issues I spotlighted in 1.2. However, only recently have methods to tune gene expression in mammalian cells in a reliable manner emerged. Thus, the advent of these new methodologies creates a timely opportunity to explore the approach of fine-tuning CAR expression.

### 1.3 STRATEGIES TO TUNE GENE EXPRESSION LEVELS IN MAMMALIAN CELLS

Eyquem and colleagues' work spotlights how crucial CAR expression is in CAR T efficacy. Even more, it reiterates how CAR expression represents a potential strategy to address the limitations previously discussed. Thus, I decided to focus my research efforts on further elucidating and characterising the effects of tuned CAR expression. I will now consider various technologies that can be used to tune CAR expression levels and highlight the miSFITs technology, the technology I used for my studies in CAR expression.

One of the first systems to tune protein expression was reported by Ferreira and colleagues and involves synthetic upstream open reading frames (uORFs) [383]. These are RNA leader sequences that are included upstream of transgenes of interest within recombinant DNA. These sequences function by changing the rate of ribosome assembly and translation. They are composed of different translation initiation sequences (TIS) (e.g., UUU, ACC, etc.). Ferreira and colleagues used different copy numbers and combinations of these TIS sequences (e.g., ACC/ACC/ACC/ACC, UUU/GGG, etc.) to increase the number of levels of expression possible. Thus, these uORFs suppress the translation rates of downstream genes in a sequence dependent manner. Ferreira and colleagues used this technology to effectively control transgene expression levels to 60 different levels, across different cell types [383]. This system could be used to control CAR expression levels, as a panel of uORFs Ferreira and colleagues could be introduced upstream of the CAR transgene.



**Figure 1.3: CRISPR/Cas9, a genome engineering tool harnessed for control of gene expression levels.** **A)** Cas9 and nuclease-deficient Cas9 (dCas9) protein in complex with sgRNA (single-guide RNA), demonstrating CRISPR/Cas9 as a targetable DNA-binding domain for gene editing (i) and gene regulation (ii). In gene editing (i), Cas9 generates a double-strand break that is erroneously repaired via endogenous pathways, such as non-homologous end-joining (NHEJ). In gene regulation (ii), dCas9 has point mutations introduced into its nuclease domains, abrogating cutting ability. Thus, dCas9 serves as a targetable DNA-binding domain that impedes the transcription of genes it is directed to. **B)** Using nuclease deficient Cas9 protein (dCas9), CRISPR/Cas9 can be purposed for gene regulation by appending on transcriptional repression domains Mxi1, KRAB, or SID4x to the dCas9 protein, eliciting CRISPR-interference (CRISPRi) (i). Similarly, transcriptional activator proteins (e.g. VP64 or p65AD) or systems (VPR, a tripartite-tandem fusion of transcription activators VP64, p65, Rta; SunTag; and synergistic activation mediator (SAM)) to upregulate endogenous gene expression to achieve CRISPR-activation (CRISPRa) (ii-iv). Adapted material from: Dominguez, A.A., Lim, W.A., Qi, L.S., "Beyond editing: repurposing CRISPR–Cas9 for precision genome regulation and interrogation", *Nature Reviews Molecular Cell Biology*, published 2015, Springer Nature. Copyright 2015, with permission from Springer Nature. License 5465990624894 [377]

The CRISPR/Cas9 system is a recent, revolutionary genome engineering tool that scientists have reprogrammed into a targetable DNA-binding domain, allowing for gene editing or regulation at any gene locus [193-195, 377]. It functions through the synergy of a Cas9 protein and a synthetic guide RNA (sgRNA) (Figure 1.3). Originating from a bacterial/archaeal immune system, the Cas9 protein bears nuclease functions to efficiently cleave double-stranded DNA in a targeted, sequence-specific manner. The sgRNA directs the Cas9 protein to any sequence that bears the appropriate proto-spacer adjacent motif (PAM).

In gene editing, Cas9 introduces double-strand breaks at the gene locus it is guided to [193-195].

These can be repaired by one of two endogenous DNA repair pathways: non-homologous end-joining (NHEJ) or homology-directed repair (HDR). NHEJ erroneously repairs Cas9-generated double-strand breaks, introducing short insertions/deletions (indels) or polymorphisms [193-195]. The introduction of indels can give rise to frameshifts into a gene's open-reading frame, potentially giving rise to a premature stop codon and loss of protein function. Similarly, polymorphisms change the DNA sequence, potentially changing the codon, leading to a change or loss in protein function. To harness the endogenous HDR pathway, a repair template that bears homology to the gene locus of interest can be provided [193-195]. The repair template can be designed such that nucleotide substitutions or large insertions can be introduced at the site Cas9 is directed to. Thus, CRISPR/Cas9 mediated gene editing can be harnessed to efficiently elicit gene knockouts, thereby rendering genes non-functional and eliminating the expression of the protein products. This is known as CRISPR-KO.

In gene regulation, Cas9 has point mutations introduced into its two nuclease domains, abrogating Cas9's DNA-cleaving ability (Figure 1.3a, ii) [377]. This is referred to as nuclease deficient Cas9 (dCas9). Thus, when coupled with a sgRNA, dCas9 serves purely as a programmable DNA-binding domain. dCas9 can then be coupled to different transcriptional effectors to regulate the expression of gene loci it is directed to. In CRISPR interference (CRISPRi), dCas9 interferes with transcription of genes by disrupting transcription factor binding or blocking RNA polymerase from progressing (Figure 1.3a, ii) [377]. This results in the silencing of gene expression. This silencing can be enhanced by fusing transcriptional repressors to dCas9 such as Max-interacting protein 1 (Mxi1), Kruppel-associated box (KRAB), or 4 repeats of mSin3 interaction domains (SID4X) (Figure 1.3b, i) [377-379].

Similarly, in CRISPR-mediated gene activation (CRISPRa) (Figure 1.3b, ii-iv), dCas9 is fused to transcription activators to upregulate the genes it is targeted to [377]. These activators include VP64, multiple repeats of herpes simplex VP16 activation domain or p65AD, nuclear factor-kB transactivating subunit activation domain (Figure 1.3b, ii). These activators have been combined with Rta, Epstein-Barr virus R transactivator, to create a fusion protein known as VPR (VP64-p65-Rta) to enhance gene activation [377, 380-382] (Figure 1.3b, iii). Further systems have been developed to

improve gene activation. For example, SunTag system features dCas9 fused to tandem GCN4 peptide repeats, and a single chain variable fragment (scFv) GCN4 antibody fused to superfolder-GFP (sfGFP) and VP64 [377, 379, 382] (Figure 1.3b, iii). Thus, multiple copies of VP64 are recruited to dCas9 by the GCN4 repeats, further improving activation. In the Synergistic Activation Mediator (SAM) system, hairpin aptamers are introduced into the guide RNA scaffold [377, 381-2] (Figure 1.3b, iv). These aptamers recruit MS2 bacteriophage coat proteins (MCP). The SAM system appends transcriptional activators p65AD and HSF1, heat shock factor 1, to these MCPs, thus recruiting them to dCas9-VP64 for enhanced gene upregulation [377, 381-2].

Work by Jost and colleagues harnessed CRISPRi to titrate gene expression in mammalian cells [196]. In their system, they introduced single mismatches into the guide RNA's spacer sequence, including all three nucleotide alternate substitutions. This changed the complementarity between gRNA and the target gene. They further introduced single mismatches into the guide RNA's constant sequence, also including all three nucleotide alternate substitutions, changing the level of binding between sgRNA and dCas9. Synergising these mismatches, Jost and colleagues used this developed library of attenuated sgRNAs to tune expression levels up to 100 levels of genes implicated with cell growth.

This system could be adapted to tune CAR expression levels. For example, the CAR transgene could be introduced into T cells alongside dCas9 and an optimised library of attenuated sgRNAs targeting the CAR. However, this system has the notable drawback of constitutive expression and activity of dCas9, giving rise to immunogenicity concerns [197-200] and off-targeting [201-206] that would likely need to be optimised.

Another system described by Chassin and colleagues involved destabilising tags, known as degrons [207-209]. In their system, they use ubiquitin (Ub), a highly conserved, 76-amino-acid polypeptide modification. It is attached to proteins by components of the Ub-proteasome system, including the Ub-activating enzyme (E1), Ub-conjugating enzyme (E2), and Ub ligase (E3) [210]. When Ub is linked to a protein, the protein is targeted to the proteasome, following the Ub-fusion degradation (UFD) pathway or N-end rule pathway. The N-end rule states that the N-terminal amino acid of a

protein determines its half-life [209, 211, 212], presenting a method to control protein levels. Chassin and colleagues harnessed this rule to develop a library of Ub tags that tuned expression to stepwise levels. This library included a set of 20 Ub tags by varying the N-terminal amino acid of the Ub tag (e.g. UbR, UbP, UbW, etc), giving rise to varying levels of expression. They included an additional 10 Ub tags (e.g. UbVV, UbAR, etc) that targeted proteins to the UFD pathway [213]. However, they introduced a mutation into the C-terminal isopeptidase site, precluding recognition by the deubiquitinating enzymes. This ultimately resulted in the lowest expression levels. They included four final tags harnessing the Ub-proteasome pathway for the Lac repressor spacer (lacS) [213] or a Ub-independent pathway [214, 215] (PEST, 2xPEST, etc. – polypeptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T)). Chassin and colleagues leveraged this library to tune reporter expression to 37 different levels [209].

This system has been previously suggested to tune CAR expression levels [216]. Indeed, the CAR transgene could be introduced into the Ub tag library Chassin and colleagues developed, tuning CAR expression levels. This represents a promising strategy, given the potential to develop further degradation tags using D-elements, unstructured initiation sites, or short sequences rich in acceptor lysines [208, 217, 218], creating further expression levels.

Work in the Fulga laboratory also addressed the outstanding gap in technologies to finely tune gene expression. To do so Michaels and colleagues developed a tuning system harnessing miRNAs. MicroRNAs (miRNA) are short non-coding RNAs that post-transcriptionally control gene expression levels through the recruitment of RNA-induced silencing complex (RISC) to cellular RNAs harbouring cognate miRNA response elements (MREs). Notably, the magnitude of repression is dependent on the degree of complementarity between a miRNA and its target MRE.

MicroRNA silencing-mediated fine-tuners (miSFITs) have been previously demonstrated as a robust technology to precisely and predictably control gene expression levels. miSFITs are artificial MREs in which point mutations have been introduced into the sequences, changing the degree of complementarity between miRNA and MRE, and altering their repressive strength. Michaels and colleagues characterized the mechanisms of action by which miSFITs operate, post-binding to miRNA.



On one hand, miSFITs function by degradation of RNA transcripts, mediated by Ago2-silencing or transcript destabilization. On the other, they function by also repressing translation. Most notably, the point mutations introduced into MRE sequence, tuning complementarity to miRNA, correspondingly tuned the degree of these transcriptional and translational pathways on target genes.

They harnessed this technology to tune numerous targets across multiple levels of expression. miSFITs tuned transgenes EGFP and PD-1 into 19 levels, with fold changes of 28x and 123x, respectively. Furthermore, they demonstrated tuning of the endogenous *BRCA1* locus to 17 levels. Most notably, they used the miSFITs tuning system to tune ovalbumin, an immunogenic protein used in immune response models, *in vivo*. In their testing, they used miSFITs to tune ovalbumin to four levels in B16-F10 cells, a murine cell line. They then injected these cells into mice models, allowing them to establish tumors before infusion CD8<sup>+</sup> OT-1 T cells. Tumor growth and animal survival rates were tuned as a result of tuned ovalbumin expression. Equally noteworthy, tumour infiltrating lymphocytes harvested post-injection demonstrated tuned levels of CD69, CD25, PD-1, and CTLA-4, key markers associated with T cell activation and exhaustion.

In summary, the miSFITs system is a genetically encoded tuning system that is easy and simple to integrate with genes of interest. It is comprehensively characterised to single-base resolution. Most importantly, it tuned expression levels of numerous genes both *in vitro* and *in vivo*. Taken altogether, miSFITs is a robust and reliable technology to tune gene expression levels. As such, I selected it for my investigations in tuning CAR expression.

#### 1.4 SUMMARY AND GOALS OF THIS THESIS

Thus, there is an outstanding gap in our understanding of how CAR expression levels control T cell state and function. This is an exciting outstanding challenge within the CAR-T therapy field. I reasoned that if CAR expression could be finely tuned to precise, stepwise levels, I could comprehensively characterise the relationship between CAR expression and key T cell behaviours, including activation and dysfunction. I could, therefore, use this knowledge to learn how to optimise CAR levels to

combat some of the outstanding issues within the field, such as toxicity, tonic signalling, and T cell exhaustion, while ensuring a safe and effective anti-cancer therapy.

In the work presented herein, I harness the miSFITs-tuning system to robustly tune CAR expression levels. I leverage these tuned levels of CAR expression, investigating their impact on T cell activation and transcriptome.

In chapter 3 of this thesis, I describe a T cell model in which I use miSFITs to tune CAR expression. I use this model to further demonstrate tuned CAR expression correspondingly tunes a proxy T cell activation marker. I use these observations to suggest potential ways CAR expression can control T cell activation.

In chapter 4, I move the CAR-miSFITs system from an immortalised T cell model to primary human T cells. In doing so, I demonstrate CAR tuning in a therapeutically relevant cell type. I then demonstrate how CAR expression tunes CD69, a marker of early T cell activation. I then characterise the relationship between CAR expression and CD69, reaffirming how CAR expression can control T cell activation.

Finally, in chapter 5, I use RNA sequencing to develop a transcriptomic atlas of tuned CAR expression in human, primary T cells. I use this atlas to demonstrate how CAR expression impacts different genes of the T cell transcriptome. I extend these findings, demonstrating how tuned CAR expression impacts T cell activation, dysfunction, and memory. I show how CAR expression impacts T cell subset/lineage emergence. I then embark on an unbiased analysis, identifying genes, regulons, and pathways that are strongly associated with CAR expression.

The primary aim of this thesis is to dissect how CAR expression levels impact T cell activation and transcriptome. I have sub-divided this primary aim into two main questions, as stated below:

1. Is it possible to tune CAR expression to precise levels of expression utilizing the miSFITs tuning system?
2. If so, how do CAR expression levels impact T cell activation and the transcriptome?

# 2

## Methods and Materials

For oligonucleotide sequences, flow cytometry reagents (including antibody clone names), and maps of key plasmids used in this study, see the appendix of this thesis. All oligonucleotides were purchased from Integrated DNA Technologies unless otherwise specified.

All data analysis and visualisation were conducted using Microsoft Excel, Prism, or R, unless stated otherwise.

## 2.1 DEVELOPMENT OF DN-28Z miSFITs LIBRARY

The generation of the DN-28z miSFITs library was conducted in collaboration with Elena Zanchini di Castiglioni and Lauren Overend of the Fulga laboratory, Weatherall Institute of Molecular Medicine, University of Oxford. To create a library of DN-28z CAR miSFITs I adapted the previously generated miR-17 sensitive, PD-1, programmed cell death protein 1, tuning plasmid library previously described [219]. This library comprised 19 plasmids that bore the same sequence except in the 3' UTR of PD-1, where the miSFIT was located. These constructs all contain a bi-directional promoter system. On one end, the constitutive human PGK (phosphoglycerate kinase) promoter drove the expression of nerve growth factor receptor (NGFR), used as a transduction marker. At the other end, a minimal CMV (cytomegalovirus) promoter drove the expression of PD-1. Downstream of the PD-1 sequence in the 3' UTR was the 23nt MRE sequence. The MRE sequences can be seen in Table 2.1:

| miSFIT          | Variant Sequence (5'-3') <i>Capitalised letters indicate point mutation introduced to wildtype miR-17 cognate sequence</i> |
|-----------------|----------------------------------------------------------------------------------------------------------------------------|
| V <sub>1</sub>  | ctacctg <b>A</b> actgtaagcactttg                                                                                           |
| V <sub>2</sub>  | ctacctgca <b>G</b> tga <b>C</b> gcactttg                                                                                   |
| V <sub>3</sub>  | ctacctgc <b>T</b> ctgtaagcactttg                                                                                           |
| V <sub>4</sub>  | <b>cA</b> acctgcactgtaag <b>T</b> actttg                                                                                   |
| V <sub>5</sub>  | ctacctgc <b>G</b> ctgtaagcactttg                                                                                           |
| V <sub>6</sub>  | ctacctgcactgtaagcactt <b>G</b> g                                                                                           |
| V <sub>7</sub>  | <b>A</b> tac <b>A</b> tgcactgtaagcactttg                                                                                   |
| V <sub>8</sub>  | ct <b>T</b> cctgcactg <b>A</b> aagcactttg                                                                                  |
| V <sub>9</sub>  | ctacctgcactgtaagc <b>G</b> ctttg                                                                                           |
| V <sub>10</sub> | ctacctgcact <b>C</b> taagcactttg                                                                                           |
| V <sub>11</sub> | ctacctgc <b>G</b> ctga <b>C</b> gcactttg                                                                                   |
| V <sub>12</sub> | ctacctgcact <b>C</b> taagcact <b>G</b> tg                                                                                  |
| V <sub>13</sub> | ctacc <b>C</b> gcactgtaa <b>C</b> cactttg                                                                                  |
| V <sub>14</sub> | ctacctgcactgtaag <b>AG</b> ctttg                                                                                           |
| V <sub>15</sub> | ct <b>C</b> cctgcactgtaagcactttg                                                                                           |
| Perfect 1x      | ctacctgcactgtaagcactttg                                                                                                    |
| Perfect 2x      | ctacctgcactgtaagcactttgTATctacctgcactgtaagcactttg                                                                          |
| Perfect 4x      | ctacctgcactgtaagcactttgTATctacctgcactgtaagcactttgTATctacctgcactgtaagcactttgTATctacctgcactgtaagcactttg                      |
| Scramble        | <b>cGCG</b> ct <b>T</b> c <b>C</b> c <b>C</b> g <b>GCCCCGTTCAA</b> g                                                       |

**Table 2.1:** miSFIT sequences used for DN-28z CAR tuning library

The backbone of these constructs included the lentiviral transfer vector components:

- WPRE –Woodchuck hepatitis virus post-transcriptional regulatory element
- cPPT/CTS –Central polypurine tract, recognition site for proviral DNA synthesis
- RRE –Rev response element, Rev protein binding sequence
- HIV-1 Ψ –RNA target site for packaging by nucleocapsid
- 5' LTR, truncated –truncated 5' Long Terminal Repeat from HIV-1
- 3' LTR (ΔU<sub>3</sub>) –self-inactivating 3' Long Terminal Repeat from HIV-1

I generated DN-28z CAR miSFIT lentiviral expression vectors using standard restriction cloning methods. A representative example vector of this library can be seen in the appendix (Appendix A.2, Figure A.1). The parent vector library PD-1-miSFIT was a gift from Yale Michaels. The DN-28z CAR was amplified from the pLEX-307-DN-SECOND-GEN-pDONR221 (a gift from Omer Dushek, Sir William Dunn School of Pathology) using primers DN28zCAR-F and DN28zCAR-R. I digested this PCR product, as well as the destination vector with SbfI and NheI, and ligated them

using T<sub>4</sub> DNA ligase (NEB). I transformed each ligation product into Subcloning Efficiency DH5 $\alpha$  Competent Cells (Thermo Fisher) following manufacturer's instructions. Transformants were plated overnight at 37°C on 24.5cm<sup>2</sup> ampicillin-treated LB agar plates. I recovered each plasmid using the QIAprep Spin Miniprep kit (QIAGEN) and validated successful cloning using Sanger Sequencing with primers LentiBB-CMV-seq and LentiBB-SV40pA-seq.

## 2.2 HEK 293T CELL CULTURE AND TRANSFECTION

HEK-293T cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FBS (GIBCO), referred to as complete growth media. I screened cells for mycoplasma at the outset of and throughout the project. Cells were seeded in 24 well plates, 24 hours prior to transfection, allowing them to reach 85-90% confluency on transfection day. On the day of transfection, complete growth media was replaced with DMEM, 2% FBS, referred to as transfection media. For each condition, two transfection mixtures were prepared. These contained 1  $\mu$ g of plasmid DNA and 3  $\mu$ L Polyethylenimine (PEI, 1mg/mL, SigmaAldrich) in 100  $\mu$ L Opti-MEM (GIBCO). Each mixture was applied dropwise to two wells of a 24-well plate and incubated for 24 hours. These transfections were repeated in triplicate.

## 2.3 FLOW CYTOMETRY AND FLUORESCENCE-ACTIVATED CELL SORTING

All flow-cytometry experiments were performed on the BD LSR Fortessa Analyzer, BD FACSAria Fusion, or BD FACSAriaIII (BD Biosciences) and data were analysed using FlowJo (Version 10.8.1). For experiments requiring antibody staining, cells were washed with FACS buffer (PBS (Gibco) with 5% FBS (Gibco)) before and after staining. For sorting experiments, I used BD FACSAria Fusion or BD FACSAriaIII (BD Biosciences) with a 100 $\mu$ M sorting chip. I used untransfected or untransduced cells to set sorting and analysis gates.

## 2.4 HEK293T SURFACE EXPRESSION ASSESSMENT

HEK 293T cells were harvested using 0.05% Trypsin with EDTA (Thermo Fisher Scientific). Cells were washed with FACS buffer (PBS (Gibco) with 5% FBS (Gibco) before staining with antibodies. Cells were stained with antibodies for NGFR-V<sub>450</sub> (BD Biosciences) and FLAG-APC (Milteny-Biotec), diluted in FACS buffer, by incubating with cells for 30 minutes at 4°C. Cells were analysed with flow cytometry. In downstream analysis, CAR expression was calculated by a sample's CAR expression by transfection efficiency. CAR expression was the median fluorescence intensity of FLAG-APC, and transfection efficiency was median fluorescence intensity of NGFR-V<sub>450</sub>.

## 2.5 MIRNA QUANTIFICATION

I used the miRNeasy Mini kit (Qiagen) to extract miRNA from HEK293T and untransduced Jurkats. miRNA was then reverse transcribed using a target-specific structured RT primer for miR-17 and RNU6b, which serves as a reference, housekeeping control, (TaqMan MicroRNA Assay ID 002308, 001093, Thermo Fisher) and TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher) from 100 ng total RNA input, following the manufacturers' instructions. I performed RT-qPCR using target-specific RT primers (Thermo Fisher) and TaqMan Universal Master Mix II, no UNG (Thermo Fisher) on a CFX384 real-time system (Bio-Rad). The  $\Delta\Delta C_t$  method was used to compare expression miR-17 expression of Jurkat to HEK293T by comparing the  $C_t$  of miR-17 to that of RNU6b for each sample replicate.

## 2.6 LENTIVIRUS PRODUCTION

To produce lentiviral particles in HEK-293T cells, I co-transfected each lentiviral transfer vector with pCMV-dR8.91 and pMD2.G at a ratio of 1.5:1:1 using Polyethylenimine (PEI, 1 mg/mL, Sigma-Aldrich), as described previously. After 24 hours, I exchanged the transfection media (DMEM (Gibco), 2% FBS (Gibco) ) with full media (DMEM (Gibco), 10% FBS(Gibco)). I collected and filtered (0.22

uM filter, Millipore) viral supernatant 24 hours later and stored it at -80°C until transduction.

## 2.7 JURKAT T CELL CELL CULTURE AND TRANSDUCTION

Jurkat T cells used in this study are a Jurkat cell line developed by the Steinberger lab of the Medical University of Vienna and described in Jutz et al 2017 [220]. In brief, Jurkat T cells of the E6.1 clone were transduced with pSIRV-NF-kB-eGFP to stably integrate a synthetic reporter that produces eGFP as a proxy for NFkB activity. This reporter contains NF-kB response elements that NF-kB transcription factor complexes can bind to. These response elements are upstream of a minimal promoter that drives the expression of EGFP. Thus, these Jurkats, when activated by TCR-like stimulation, will express EGFP, reporting the activity of NF-kB signalling. These cells were received in collaboration with the Dushek Laboratory of the Sir William Dunn School, University of Oxford. Jurkats were grown in Roswell Park Memorial Institute 1640 with GlutaMAX medium (RPMI, Gibco) supplemented with 10% FBS (Gibco).

For DN-28z CAR RNA transcript measurement, an optimised pool of lentivirus encoding the entirety of DN-28z CAR miSFIT was generated. Lentivirus for each DN-28z CAR miSFIT was separately generated as described above and respective functional viral titers were determined. Lentivirus was pooled for equal functional titers between each miSFIT variant. This was to ensure each CAR-miSFIT construct would transduce at equal rates, giving rise to a population of cells in which each miSFIT would be equally represented (1x Perfect transduced into 500 cells, 2x Perfect transduced into 500 cells, ..., V15 transduced into 500 cells, and Scramble transduced into 500 cells).

To generate miSFIT cell lines, I transduced cells at low multiplicity of infection (MOI) (below 10% transduced), waited 5 days, and selected stably transduced cells by FACS, sorting on NGFR positive cells, using BD FACSAria Fusion.



## 2.8 gDNA AND cDNA LIBRARY PREPARATION AND HIGH-THROUGHPUT SEQUENCING

I used the All Prep DNA/RNA Mini kit (Qiagen) to simultaneously extract genomic DNA (gDNA) and mRNA from Jurkats transduced with the DN-28z CAR-miSFITs library. After performing a genomic DNA wipe-out step (TURBO DNA free, Thermo Fisher), cDNA was generated from mRNA using the QuantiTect Reverse Transcription kit (Qiagen) following the manufacturer's instructions. To create amplicon libraries for high-throughput sequencing, the miSFIT sequence and a short flanking region were PCR amplified using the primers bi-dir-Miseq-F and bi-dir-Miseq-R. For gDNA and cDNA, I used Phusion High-Fidelity PCR Master Mix with GC Buffer (NEB) and the following cycling conditions: initial denaturation (98°C for 3 minutes), 25 amplification cycles (98°C for 10s, 60°C for 12s, 72°C for 12s), and final extension (72°C for 10 minutes).

These initial PCR products were gel-purified using the QIAquick Gel Extraction Kit (Qiagen). I diluted the recovered product between 10 and 30 fold depending on band intensity. To make amplicon libraries compatible with Illumina machines, I performed a second PCR to append TruSeq index sequences and p5/p7 adapters to each amplicon. I used a dual barcoding strategy where a unique combination of forward and reverse index primers were assigned to each biological sample. I performed the PCRs with Phusion High-Fidelity PCR Master Mix with GC Buffer (NEB), using the following cycling conditions: initial denaturation (98°C for 30 seconds), 13 amplification cycles (98°C for 10s, 62°C for 10s, 72°C for 10s), and final extension (72°C for 5 minutes). I used Agencourt AMPure XP beads (1X, Beckman Coulter) at 1X to purify the amplicon libraries. The libraries were quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher). Samples were pooled and sequenced (150bp PE) sequencing on the MiSeq v3 (Illumina).

## 2.9 HIGH-THROUGHPUT SEQUENCING DATA ANALYSIS

High-throughput sequencing data were analysed using R (version 4.2) and all scripts are available upon request. After inspecting the quality of sequencing data with FastQC, I used the Biostrings package (version 2.65.3) to trim reads down to the MRE and subsequently count the occurrence of

each type of miSFIT in all amplicon libraries. I calculated the transcript abundance for each variant by dividing its read count in the cDNA library by its read count in the respective gDNA library.

#### 2.10 JURKAT DN-28Z CAR - miSFIT GENOMIC INTEGRATION VALIDATION

I used PureLink Genomic DNA Mini kit (Thermo Fisher) to extract genomic DNA (gDNA) from the Jurkat miSFIT cell lines. The miSFIT sequence and a short flanking region were PCR amplified using the primers J-CARgTransd-F and J-CARgTransd-R. I performed PCRs with Phusion High-Fidelity PCR Master Mix with GC Buffer (NEB) and the following cycling conditions: initial denaturation (98°C for 3 minutes), 25 amplification cycles (98°C for 30s, 64°C for 25s, 72°C for 30s), and final extension (72°C for 5 minutes). I validated the size of these PCR products by gel electrophoresis. I gel-purified using the QIAquick Gel Extraction Kit (Qiagen). I sent these purified PCR products for Sanger Sequencing using J-CARgTransd-seq.

#### 2.11 JURKAT CAR EXPRESSION MEASUREMENT

Jurkat cell lines were washed with FACS buffer (PBS (Gibco) with 5% FBS (Gibco) before staining with antibodies. Jurkats were stained with antibodies for NGFR-V450 (BD Biosciences) and FLAG-APC (MiltenyBiotec), diluted in FACS buffer, by incubating with cells for 30 minutes at 4°C. Cells were analysed with flow cytometry. In downstream analysis, CAR expression was calculated by median fluorescence intensity of FLAG-APC.

#### 2.12 JURKAT NFkB-EGFP ACTIVATION CO-CULTURE AND MEASUREMENT

To study the effects of tuned CAR expression on T-cell activation, I co-cultured my Jurkat DN-28z CAR-miSFIT cell lines with target cells, CHO-K1+hCD58+HLA-A2.

Target cells used in these co-cultures were CHO-K1+hCD58+HLA-A2 cells. This is a variant of Chinese Hamster Ovary K1 (CHO-K1) cells that were transduced to expression human CD58 and HLA-A\*02. HLA-A\*02, human leukocyte antigen A\*02, is an MHC-I (major histocompatibility

complex I) molecule that binds peptide fragments and displays them on the cell surface for recognition by T cells. CD58, also known as lymphocyte function-associated antigen 3, is a cell adhesion molecule expressed on antigen-presenting cells. Its function is to facilitate and strengthen the binding of a T cells to its target cell. It accomplishes this by binding to CD2 on the T cell. Thus, in this system, hCD58 strengthens the interactions between CAR and HLA-A\*02:peptide.

Chinese hamster ovary K1 (CHO-K1) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FBS (GIBCO). I screened cells for mycoplasma at the outset of and throughout the project.

30,000 CHO-K1 cells were seeded in 96 well plates, 24 hours prior to co-culture. On the day of co-culture, DMEM, 10% was replaced with Opti-MEM containing serial diluted concentrations, ranging from 0 nM to 20 nM, of NY-ESO-1<sub>157-165</sub> 9V peptide. CHO-K1 cells were incubated with 9V peptide for 90 minutes for peptide pulsing. 9V peptide medium was aspirated and DN-28z CAR miSFITs Jurkat cell lines were added to each well at a 2 CAR-T : 1 CHO-K1 ratio. Co-cultures were incubated for 12 hours and harvested using 0.05% Trypsin with EDTA (Thermo Fisher Scientific). Cells were washed with FACS buffer (PBS (Gibco) with 5% FBS (Gibco)) before and after antibody staining for NGFR (BD Biosciences). Cells were stained with antibody, diluted in FACS buffer, by incubating with cells for 30 minutes at 4°C. Cells were analyzed with flow cytometry.

### 2.13 CD8+ T CELL ISOLATION, TRANSDUCTION, AND CAR SURFACE EXPRESSION MEASUREMENT

Human CD8+ T cells were received in collaboration with Jesus Farfan Siller and Omer Dushek of the Sir William Dunn School of Pathology, University of Oxford. CD8 T cell isolation and transduction were previously described, conducted by Jesus, and summarised below [221-223].

Human CD8+ T cells were isolated from four donor leukocyte cones purchased from the National Health Service's (UK) Blood and Transplantation service. Isolation was performed using negative selection. Briefly, blood samples were incubated with Rosette-Sep Human CD8+ enrichment cocktail

(Stemcell) at 150 µl/ml for 20 minutes. This was followed by a 3.1 fold dilution with PBS before layering on Ficoll Paque Plus (GE) at a 0.8:1.0 ficoll to sample ratio. Ficoll-Sample preparation was centrifuged at 1200g for 20 minutes at room temperature. Buffy coats were collected, washed, and isolated cells counted. Cells were resuspended in complete primary T cell media (RPMI (Thermo Fisher) supplemented with 10% FBS (Gibco), 100 Units/ml penicillin, 100 µg/ml streptomycin (Gibco)) with 50 U/ml of IL-2 (PeproTech) and CD3/CD28 Human T-activator Dynabeads (Thermo Fisher) at a 1:1 bead to cell ratio. Isolated human CD8+ T cells were cultured at 37°C and 5% CO<sub>2</sub>.

On the following day, 1 million cells were transduced using lentivirus titrated for a low multiplicity of infection (below 15% transduced). On days 2 and 4 post-transduction, 1 ml of media was exchanged, and IL-2 was added to a final concentration of 50 U/ml. Dynabeads were magnetically removed on day 5 post-transduction. CAR T cells were further cultured at a density of 1 million cells/ml and supplemented with 50 U/ml IL-2 every other day.

Day 8 post-transduction, a subpopulation of CAR Ts were harvested to measure DN-28z CAR expression levels. 500,000 CAR T cells were harvested, washed with FACS buffer (PBS (Gibco) + 5% FBS (Gibco)) before and after staining. T cells were stained with NY-ESO-1 9V PE-conjugated tetramer (produced in-house in the Dushek laboratory using refolded HLA\**A0201* with NY-ESO-1 9V and streptavidin-PE [Bio-Rad AbD Serotec or BioLegend]). Cells were stained with antibody, diluted in FACS buffer, by incubating with cells for 30 minutes at 4°C. CAR expression was measured using flow cytometry. In downstream analysis, CAR expression was calculated by geometric median fluorescence intensity of NY-ESO-1 9V-PE. CAR expression was normalised on a donor basis by dividing by Scramble control expression.

#### 2.14 CD8+ T CELL ACTIVATION CO-CULTURE AND CD69 MEASUREMENT

To once again simulate antigen-binding induced activation, I once again used my synthetic antigen presentation system that featured CHO-K1+hCD58+HLA-A\*02 as target cells.

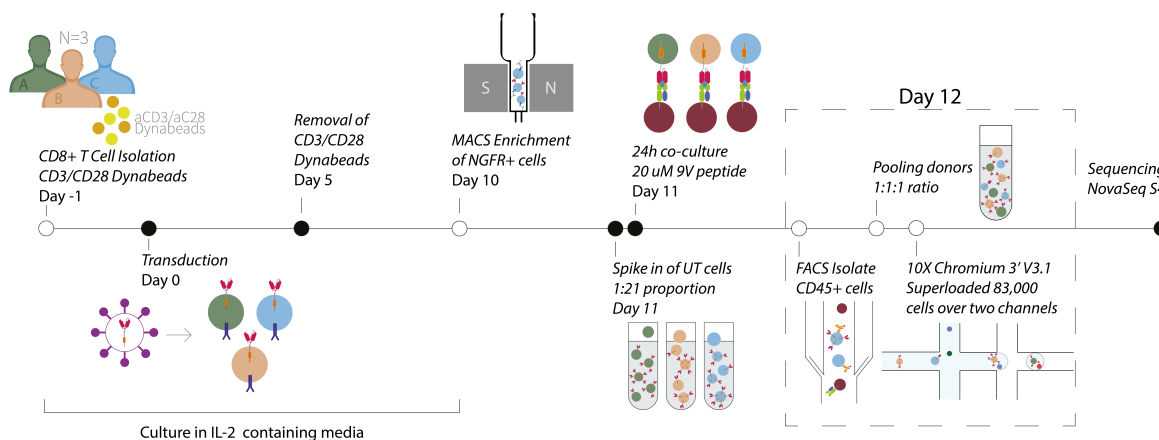
CD8 T cells were isolated, transduced, and expanded as described above. On day 10 post-transduction,

T cells were enriched for transduced cells using magnetically activated cell sorting (MACS) and CD271 (LNGFR, low-affinity, Nerve Growth Factor Receptor) MicroBead kit (MiltenyiBiotec) according to manufacturer's instructions. Briefly, cells were resuspended in MACS buffer (PBS (Gibco) + 2% FBS (Gibco), degassed before use) and magnetically labelled by incubating with CD271 microbeads (Miltenyi Biotec) for 15 minutes at 4°C. Cells were washed after staining and resuspended in MACS buffer. Cells were applied to LS columns (Miltenyi Biotec) on QuadroMACS separator magnet (Miltenyi Biotec). The column was washed 3x with MACS buffer before being removed from magnet, and cells were plunged out. Enriched CAR-T Cells were resuspended in complete primary T cell media without IL-2 supplementation and rested overnight.

The same day of MACS enrichment, 180,000 CHO-K1 cells were seeded in 24 well-plates. On Day 11, post-transduction, co-cultures were conducted as described above. However, only two peptide concentrations of NY-ESO-1<sub>157-165</sub> 9V peptide were used: 1nM and 20,480nM. On Day 12, post-transduction, cells were washed with FACS buffer (PBS (Gibco) with 5% FBS (Gibco)) before and after ZombieGreen (BioLegend) and antibody staining for CD45-BV421 and CD69-AF647 (BioLegend). Cells were stained with ZombieGreen, diluted in PBS (Gibco), at room temperature for 30 minutes, following manufacturer's instructions. Antibodies for CD45-BV421 and CD69-AF647 subsequently were added, diluted in FACS buffer, and incubated with cells for 30 minutes at 4°C. ZombieGreen served as a LIVE/DEAD stain. CD45 served to discern CAR Ts from any residual CHO-K1 cell. Cells were analysed with flow cytometry. In downstream analysis, CD69 expression was calculated by geometric median fluorescence intensity of CD69-AF647. CD69 expression was normalised on a donor basis by dividing by Scramble control expression.

#### 2.15 CD8<sup>+</sup> T CELL SINGLE-CELL RNA SEQUENCING

I used 10X Genomics' Chromium Single Cell 3' Gene Expression v3.1 kit and workflow for single-cell RNA-sequencing (scRNAseq) library creation. My overall protocol is shown in Figure 2.1.



**Figure 2.1: Overview of protocol and workflow to generate single-cell RNA-sequencing libraries.**

### 2.15.1 CREATION OF EMPTY TRANSDUCTION PLASMID CONTROL

To have a negative transduction control that could be detected with a miSFIT sequence, I cloned an empty expression vector. I used an online Random DNA Sequence generator to generate a DNA sequence of equal length to the DN-28z CAR gene (<http://www.faculty.ucr.edu/~mmaduro/random.htm>). I manually inspected this sequence, mutated any potential transcription start sites and encoded frequent STOP codons throughout the open reading frame (ORF), and ordered it as gBlock Gene Fragment (IDT). I linearised and removed the NY-ESO-1 DN-28z CAR sequence from the NY-ESO-1-2ndGenCAR-WT-Mir17RE-1x plasmid by digesting with restriction enzymes SbfI (NEB) and NheI (NEB). I combined the gBlock with the linearised plasmid using the In-Fusion HD Cloning System (Takara Clontech), following the manufacturer's instructions. I transformed the In-Fusion product into Subcloning Efficiency DH5a Competent Cells (Thermo Fisher). After using QIAprep Spin Miniprep kit (QIAGEN) to isolate and purify the resulting plasmid, I validated successful cloning using Sanger Sequencing with primers LentiBB-CMV-seq and LentiBB-SV40pA-seq. I linearised and removed the 1x Perfect miSFIT sequence from this plasmid using restriction enzymes NheI (NEB) and AgeI (NEB). A new MRE insert was generated by synthesizing and annealing synthetic, overlapping oligonucleotides comprising the MRE and appropriate sticky overhangs. I ligated the insert to

the linearised vector for 1 hour at room temperature using T4 DNA Ligase (NEB). I transformed the ligation product into Subcloning Efficiency DH5a Competent Cells (Thermo Fisher). I named the resulting product Empty-MRE.

#### 2.15.2 CD8 T CELL ISOLATION, BULK TRANSDUCTION, AND EXPANSION

Lentivirus for each DN-28z CAR miSFIT was individually generated as described above and respective functional viral titers were determined. Lentivirus was pooled for equal functional titers between each miSFIT variant. This was to ensure each CAR-miSFIT construct would transduce at equal rates, giving rise to a population of cells in which each miSFIT would be equally represented (1x Perfect transduced into 500 cells, 2x Perfect transduced into 500 cells, etc, V15 transduced into 500 cells, and Scramble transduced into 500 cells).

CD8+ T cells were isolated from three donors, as previously described above in 2.12. On day 0, isolated T cells were transduced with an optimised pool of lentivirus generated as described above in 2.12. Post-transduction, T cells were expanded, as previously described above in 2.12. Day 10 post-transduction, T cells were MACS enriched, using CD271 MACS microbead kit (Miltenyi Biotec), as previously detailed above in 2.12. Post-MACS enrichment, MACS flow-through (containing unlabeled, untransduced cells) was spun down, counted, and spiked in to enriched T cell populations at a 1:21 ratio. This was done to have negative, untransduced control T cells within my population. T cells were rested overnight.

On the same day of MACS enrichment, 900,000 CHO-K1 cells were seeded in 6 well plates. Co-cultures were conducted as described above, being initiated on Day 11 post-transduction. However, only 20,480nM peptide concentration of NY-ESO-1<sub>157-165</sub> 9V peptide were used.

#### 2.15.3 RNA LIBRARY PREPARATION

On Day 12 post-transduction, cells were washed with FACS buffer (PBS (Gibco) with 5% FBS (Gibco)) before and after ZombieGreen (BioLegend) and antibody staining for CD45-BV421 (BioLegend). Cells were stained with ZombieGreen, diluted in PBS (Gibco), at room temperature for 30 minutes,

following manufacturer's instructions. Cells were stained with CD45-BV421 antibody, diluted in FACS buffer, at 4°C for 30 minutes. CD45+ cells were selected by FACS using BD FACSAria III and BD FACSAria Fusion. CD45 served to discern CAR T cells from any residual CHO-K1 cell. Furthermore, selection on CD45 included both untransduced cells and enriched, CAR T cells. Post-FACS sorting, a subpopulation of each donor's, FACS-sorted T cells were saved for downstream bulk RNA-sequencing library preparation.

Each donor's FACS-sorted T cells were then pooled at a 1:1:1 ratio. This pool was diluted and approximately 84,000 cells were super-loaded across two Chromium 10X Chip channels (10X Genomics). 10X Chromium Next Single Cell 3' v3.1 kit (10X Genomics) was used for single-cell capture, cDNA synthesis, and single-cell RNA-sequencing (scRNA-seq) library construction, following manufacturer's instructions. scRNA-seq library was quality-controlled using TapeStation and quantified using Qubit. scRNA-seq library was sequenced on NovaSeq 6000 S4 (150bp PE).

I used cDNA generated from the 10X single-cell reaction to create PCR amplicon libraries that captured the 10X cell barcode and miSFIT sequence. I designed my library preparation such that the 10X cell barcode would be captured in Read1 of sequencing, while the miSFIT sequence would be captured in Read2.

Primers 10X-CARmiSFIT-F and 10X-CARmiSFIT-R were used to PCR amplify the miSFIT sequence and 3' captured 10X cDNA. I used NEBNext Ultra II Q5 Master Mix (NEB) and the following cycling conditions: initial denaturation (98°C for 30 seconds), 30 amplification cycles (98°C for 10s, 65°C for 12s, 72°C for 12s), and final extension (72°C for 10 minutes). Primers 10x-EmptymiSFIT-F and 10x-EmptymiSFIT-R were used to separately capture the previously generated Empty miSFIT. NEBNext Ultra II Q5 Master Mix (NEB) and the following cycling conditions: denaturation (98°C for 30 seconds), 25 amplification cycles (98°C for 10s, 69°C for 12s, 72°C for 12s), and final extension (72°C for 10 minutes). I used gel electrophoresis to validate the size of the resulting PCR products.

These initial PCR products were gel-purified using the QIAquick Gel Extraction Kit (Qiagen). I further purified these products using Agencourt AMPure XP beads (1X, Beckman Coulter) at 1X. To make amplicon libraries compatible with Illumina machines, I adopted the dual-barcoding strategy as



previously described in 2.5.

To append on TruSeq index sequences and p5/p7 adapters to the 10X CAR-miSFIT libraries, I performed PCRs using TruSeq-501-10XCAR and TruSeq-701-10XCAR with NEBNext Ultra II Q5 Master Mix (NEB) and the following cycling conditions: denaturation (98°C for 30 seconds), 13 amplification cycles (98°C for 10s, 61°C for 12s, 72°C for 12s), and final extension (72°C for 10 minutes). For the Empty library, I used TruSeq-501 and TruSeq-701 with NEBNext Ultra II Q5 Master Mix (NEB) and the following cycling conditions: denaturation (98°C for 30 seconds), 13 amplification cycles (98°C for 10s, 61°C for 12s, 72°C for 12s), and final extension (72°C for 10 minutes).

I used Agencourt AMPure XP beads (1X, Beckman Coulter) at 1X to purify the amplicon libraries. The libraries were QC using 2100 Bioanalyzer (Agilent) and quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher). The 10X-CAR-miSFIT library was pooled with the 10X-Empty-miSFIT library at a 1:21 ratio. The final library was sequenced (300bp PE) on the MiSeq v3 (Illumina).

For each donor, separate bulk RNA-sequencing libraries were generated for downstream genetic demultiplexing. I used All Prep DNA/RNA Mini kit (Qiagen) to simultaneously extract genomic DNA (gDNA) and mRNA from 1x10<sup>6</sup> FACS-sorted T cells. After genomic DNA wipe-out step (TURBO DNA free, Thermo Fisher), I created RNA-sequencing libraries using SMART-Seq v4 Ultra Low Input RNA Kit (Takara Bio) and Nextera XT DNA Library (Illumina) following manufacturer's guidelines. Libraries were QC using 2100 Bioanalyzer (Agilent) and quantified using Qubit (Thermo Fisher). Libraries were sequenced on NextSeq 500 (75bp PE).

## 2.16 SINGLE-CELL RNA-SEQUENCING COMPUTATIONAL ANALYSIS

### 2.16.1 CUSTOM HUMAN GENOME FEATURING DN-28Z CAR-MISFIT TRANSCRIPT

For all RNA-sequencing analysis, I used a single custom human genome reference that featured the DN-28z CAR miSFIT transcript. I used Genome Reference Consortium Human Build 38 patch release 13 genome assembly (GRCh38.p13) and appended on the DN-28z CAR-miSFIT transcript using Cellranger's 'mkref' function. I refer to this custom genome as GRCh38.p13.DN28zmiSFITs.

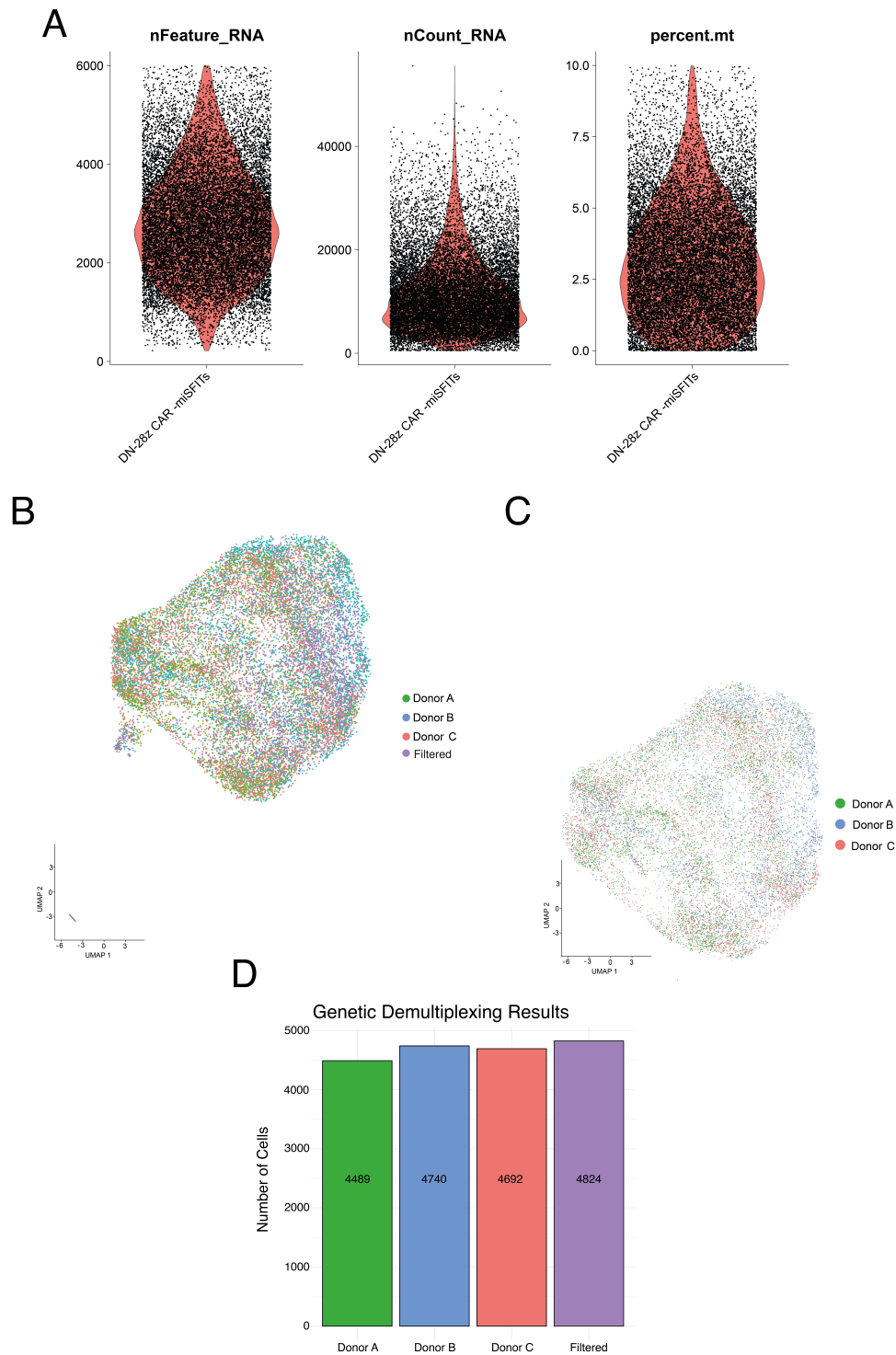
### 2.16.2 PREPROCESSING AND QC OF SINGLE-CELL RNA-SEQUENCING DATA

Single-cell RNA-seq fastq files were demultiplexed using Cellranger (v6.1.2 10X Genomics) and mapped to the previously described GRCh38.p13.DN28zmiSFITs genome. 20,171 estimated cells were obtained with mean reads per cell of 141,178, median genes per cell of 2,653, median UMI counts per cell of 9,768, and a total number of genes detected was 16,524. 96.2% of reads had valid barcodes with a Q30 of 94.7% and 94.1% of the reads mapped confidently to the human genome. Downstream analysis and visualization were carried out using Seurat (v4.1.1) in R [224-227]. Matrices were filtered to remove barcodes with fewer than 200 genes (nFeature\_RNA), more than 6000 genes expressed and carried a high percentage of UMIs from mitochondrial features (percent.mt, greater than 10%) (Figure 2.2a).

EmptyDrops (v1.17.1, [228]) was used on Seurat-filtered barcodes to identify and filter out empty droplets. scDblFinder (v1.11.3, [229]) was used on EmptyDrops-filtered barcodes to identify and filter out any doublets. Outstanding barcodes were then subjected to genetic demultiplexing and miSFIT demultiplexing pipelines.

### 2.16.3 GENETIC DEMULTIPLEXING

The genetic demultiplexing pipeline was carried out in collaboration with Santiago Revalle of the Wellcome Centre Human Genetics of the Nuffield Department of Medicine, University of Oxford. Genetic demultiplexing was previously described, conducted by Santiago, and summarized as follows [230]. Donor bulk RNA-sequencing fastq files were quality-control checked with FastQC. Adaptor sequences were trimmed using TrimGalore (v0.6.5, Babraham Institute) and aligned reads to custom reference genome (GRCh38.p13.DN28zmiSFITs) using STAR (2.7.3, [231]). Minor modifications were made to the variant calling pipeline to incorporate metadata into the BAM alignment files and to produce a block gVCF file. GATK variant calling pipeline (v4.1.7) was applied to BAM alignment files to identify sequence variants. cellSNP (v0.3.1, [232]) and Vireo (v0.4, [233]) were used to genotype and demultiplex filtered barcodes.



**Figure 2.2: Preprocessing and genetic demultiplexing of single-cell gene expression matrix.** **A)** Quality control filtering of single-cell gene expression matrix. nFeatureRNA, nCountRNA, and percent mitochondrial UMI reads were used as QC measures. **B)** UMAP visualisation of genetic demultiplexing results. Filtered labelled cells refer to empty droplets or multiplets identified. **C)** UMAP visualisation of donor distribution. **D)** Demultiplexed cells per donor.

The EmptyDrops, scDblFinder, and the genetic demultiplexing pipelines identified 4,840 empty droplets, and multiplets, and interpatient doublets, which were subsequently filtered out in downstream analysis (Figure 3.2.2b,c). This resulted in 13,921 single cells being used for downstream analysis. All three donors (A, B, and C) distributed evenly across the UMAP visualisation (Figure 3.2.2c), and similar numbers of cells demultiplexed to each donor (Figure 3.2.2d).

#### 2.16.4 MISFIT DEMULTIPLEXING

10X-PCR amplicon library capturing 10X cell barcode, DN-28z CAR, and miSFIT sequences were analysed using R (version 4.2) and all scripts are available upon request. After inspecting the quality of sequencing data with FastQC, I used ShortRead (v1.55,[234]), Biostrings package (v2.65.3), stringr (v1.4.1), and dplyr (v1.0.10) to write an algorithm that conducted the following steps:

1. Searched for the Seurat QC-filtered 10X cell barcodes in Read 1 of the resulting PCR amplicon fastq files
2. Counted the number of times any miSFIT sequence appeared in the respective Read2
3. For a cell barcode with any detected miSFIT counts, considers the two miSFITs with the highest counts (referred to as M1, miSFIT with the highest, and M2, second highest, from hereon) and assigns a miSFIT based on the following requirements, designed to remove ambiguous cases and intra-patient multiplets:
  - (a) M1 does not equal M2
  - (b) M1 exceeds a minimum read count of 25 (Figure 2.3 a,b)
  - (c) The ratio of M1 to M2 exceeds the minimum factor threshold of 3 (Figure 2.3 c,d)
  - (d) Should any of the above requirements be failed, the cell was unlabelled and the cell barcode discarded

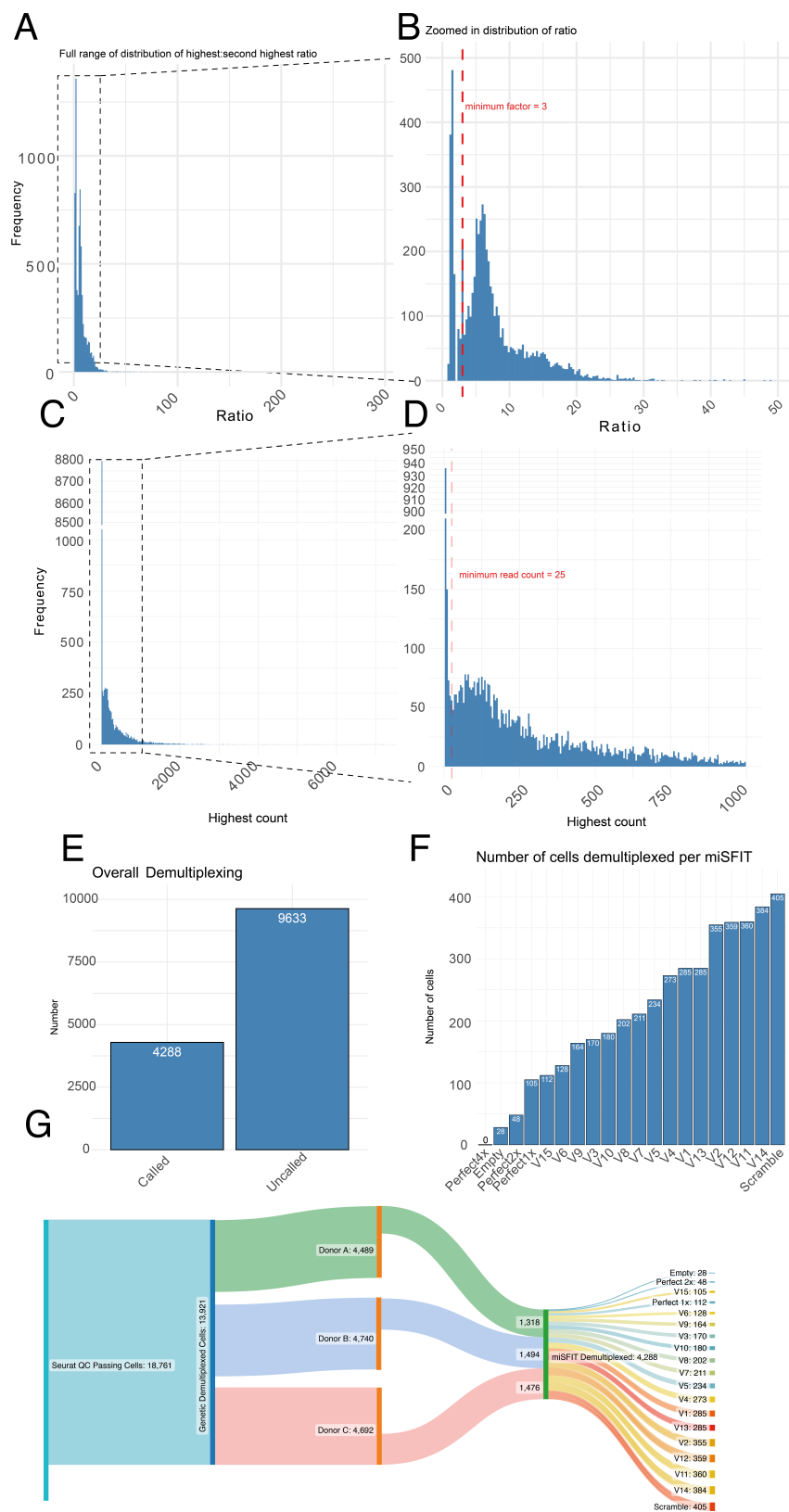


Figure 2.3: miSFIT demultiplexing algorithm. Continued on the following page.

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**Figure 2.3: miSFIT demultiplexing algorithm.** *Continued from the previous page.* miSFIT demultiplexing algorithm. **A,B)** Distribution of miSFIT read count. **C,D)** Distribution of miSFIT calling ratio. **E,F)** Results of demultiplexing in **E)** total, **F)** per miSFIT. **G)** miSFIT demultiplexing incorporating genetic demultiplexing filtering.

Of the 18,761 Seurat QC-passing cell barcodes, 6,420 barcodes (34%) were demultiplexed to a miSFIT (Figure 2.3e). 628 cells, the highest number of cells of all miSFITs, demultiplexed to the scramble miSFIT control, while zero cells demultiplexed to the Perfect4x miSFIT. 34, 57, and 153 cells demultiplexed, respectively, to Empty, Perfect2x, and Perfect1x miSFITs (Figure 2.3f). When factoring in the genetic demultiplexing pipeline filtering, 4,288 cells were demultiplexed to a miSFIT, 67% of the total number of barcodes associated with a miSFIT (Figure 2.3g).

#### 2.16.5 POST DEMULTIPLEXING AND QC ANALYSIS

Donor-identified and miSFIT-identified barcodes were annotated into single-cell expression matrices. Cells that were not donor-identified were filtered out. Post filtering, 13,921 barcodes were used for analysis. Expression values for the total UMI counts per cell were log-normalized and scaled. Highly variable genes were computed before performing principal component analysis (using 50 principal components). Linear dimensional reduction was conducted (resolution = 0.5, dims = 1:50). 9 cell clusters were obtained and visualized on UMAP (Figure 2.4a).

Gene expression levels were calculated according to the following calculation, as per Seurat's standard analysis pipeline:

$$Gene\ Expression\ Level = \ln\left(\left(\frac{GOI_{UMI\ Count}}{Total_{UMI\ Count}} * 10,000\right) + 1\right)$$

*where  $GOI_{UMI\ Count}$  refers to a gene of interest's UMI count within a cell*

*where  $Total_{UMI\ Count}$  refers to the total UMI count of the same cell*

Any mention of a gene expression level within this thesis, both within the text and in figures, uses this base calculation.

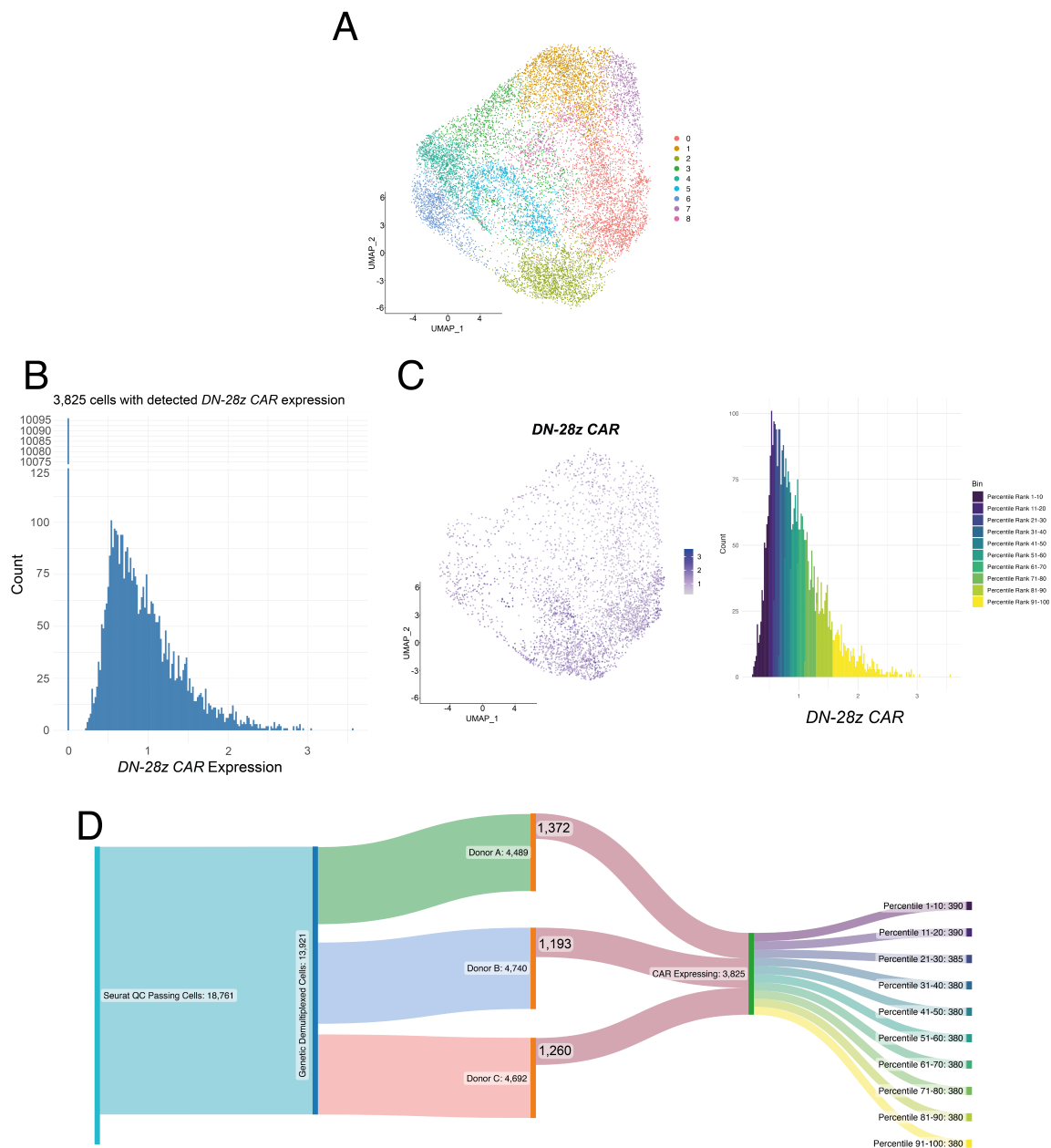
For downstream analysis, unless stated otherwise, the single-cell expression matrix was subsetted to only include cells with detected DN-28z CAR expression (Figure 2.4b,c). This resulted in 3,825 cells being in the single cell expression matrix (Figure 2.4b,c). Cells were subsequently annotated according to their percentile rank in DN-28z CAR expression. 10 bins were created based on DN-28z CAR percentile rank: 1-10, 11-20, 21-30, 31-40, 41-50, 51-60, 61-70, 71-80, 81-90, 91-100, and cells were binned accordingly (Figure 3.2.4b,c,d). These bins are hereafter referred to as CAR expression bins.

Gene expression levels were averaged according to CAR expression bin and used for subsequent analysis, referred to as averaged expression matrix. Cells were scored using the escape package (v1.7.0) using UCell algorithm [235] for dysfunction, effector, and naïve/memory gene signatures published by Singer and colleagues [236]. Enrichment scores were averaged according to CAR expression bin. BioCarta pathways were downloaded from the Molecular Signatures Database by the misgdbR package (v7.5.1, [237]), and cells were scored using the escape package (v1.7.0) using the ssGSEA algorithm. Enrichment scores were averaged according to CAR expression bin. Mathematical modelling was conducted using the stats package (v3.6.2) and drc package (v3.0-1) and visualized using the ggplot2 package (v3.3.6).

All 13,921 cells were used in cell annotation analysis. I downloaded Zheng and colleagues' SeuratObject which compiled and integrated multiple scRNA-seq datasets for CD8<sup>+</sup> T cells [238]. I used Seurat's TransferAnchor [225] to transfer the annotations of Zheng's SeuratObject onto my single-cell expression matrix.

Regulons were computed with the SCENIC (Single-Cell rEgulatory Network Inference and Clustering) pipeline (v1.3.1 [239, 240]) using CAR expressing cells. GENIE3 (v1.19.0, [241]) was used to determine coexpression modules. hg38 transcription factor motifs provided by the Aerts lab were used to identify direct transcription factor motifs using the RcisTarget package. Regulon activity was quantified in single cells using the Area Under Curve metric built into the pipeline, calculated by AUCell (v1.19.1). Regulon specificity score was calculated as described by Suo and colleagues [242] by running calcRSS command of SCENIC.

Linear and logarithmic regression was conducted using the stats package (v4.3.0) using the averaged



**Figure 2.4: DN-28z CAR expression and binning strategy.** A) UMAP visualisation of resulting KNN-generated clusters via Seurat. B, C, D) Detected DN-28z CAR expression and percentile rank binning.



expression matrix. Genes were regressed against the DN-28z CAR. Any genes with a regression model  $R^2$  above 0.9 were used for subsequent analysis. The *infotheo* package (v1.2.0.1) was used for mutual information (MI) analysis calculations. I used a mutual information variant known as normalized mutual information (NMI). NMI is suggested to be an equivalent value to the Pearson's correlation coefficient, where an NMI of 1 is interpreted to be a perfect correlation [243]. In MI terminology, this is therefore interpreted to have a complete mutual dependence between two variables. The formula for NMI is

$$NMI = \frac{I(X; Y)}{\sqrt{H(X)H(Y)}}$$

Where  $I(X;Y)$  is the mutual information between X and Y, and H is the respective entropy of X and Y. I used the *infotheo* package using the average expression matrix to calculate the normalized mutual information of all genes against the DN-28z CAR. Genes with an NMI value of 1 were used for subsequent analysis. I used the *clusterProfiler* package (4.5.2) or the online web portal of PantherDB, release 17.0, to conduct Gene Ontology analysis for biological processes. For *clusterProfiler*, reported  $P_{adj}$  values were calculated using the Benjamini-Hochberg correction. For PantherDB, overrepresentation analysis using Fisher's Exact test, calculating for false discovery rate (FDR) was used. For this analysis, a minimum overrepresentation fold enrichment of 10 and cutoff of FDR less than 0.05 was used.

For the majority of heatmap visualizations, unless stated otherwise, gene expression, regulon activity score, or enrichment score were rescaled using *scales* package (v1.2.1). Values were rescaled from 0 to 1, where each value's respective minima was set to 0 and maxima set to 1, for ease of visualization purposes.

# 3

## Developing a T-cell model of tuned CAR expression

As laid out in the introduction of my thesis, my DPhil is guided by two main questions:

1. Is it possible to tune CAR expression to precise levels utilizing the miSFTs tuning system?
2. If so, how do CAR expression levels impact T cell activation and transcriptome?

In this subchapter, I begin by tackling the first question motivating my DPhil. To do so, I develop a library of DN-28z CAR-miSFTs plasmids. I then introduce this library via transient transfection into HEK293T cells. This experiment provides preliminary evidence of tuning CAR expression. I then stably integrate this system into a more representative cell type, an immortalized T cell line called Jurkat. In this platform, I demonstrate further compelling evidence of robust, stepwise tuning of CAR expression, answering the first question guiding my DPhil in a T cell model.

I then address the second question guiding my DPhil: what impact does tuned CAR expression have on T cell activation and transcriptome? An essential function of CAR-T cells is the ability to activate and proliferate in response to tumour antigens, creating more immune cell 'warriors' to attack cancer cells. In these T cell model studies, I focus my efforts on understanding the impact of tuned CAR expression on a T cell activation proxy using an EGFP reporter for NFkB activity. As a result of these efforts, I demonstrate that tuned CAR expression tunes this marker of T cell activation, answering the second question motivating my DPhil.

I then direct my efforts to a natural corollary to my second question: What is the relationship between T cell activation status and CAR expression levels? In this analysis, I use mathematical models to demonstrate that my T cell activation marker, NFkB-EGFP, portrays a logarithmic relationship with CAR expression.

In this subchapter, I provide foundational data that tuned CAR expression impacts T cell activation in a model system. I show that tuning CAR expression is a promising avenue to control T cell functions.

### 3.1 A PROOF OF CONCEPT

The first question guiding my DPhil is whether CAR expression levels can be robustly tuned to precise, stepwise levels. As highlighted in my introduction chapter, previous work conducted in the Fulga laboratory demonstrated a synthetic tuning system leveraging microRNAs. In brief, microRNAs (miRNA) are short non-coding RNAs that post-transcriptionally control gene expression levels through the recruitment of RNA-induced silencing complex (RISC) to cellular RNAs harbouring cognate miRNA response elements (MREs). Notably, the magnitude of repression is dependent on the degree of complementarity between a miRNA and its target MRE.

MicroRNA silencing-mediated fine-tuners (miSFITs) have been previously demonstrated as a robust technology to control gene expression levels precisely and predictably. miSFITs are artificial MREs in which point mutations have been introduced into the sequences, changing the degree of complementarity between miRNA and MRE, thus altering their repressive strength. By introducing MREs with distinct mutations downstream of a gene of interest, users can genetically encode defined expression levels of genes. This technology has previously been applied to fine tune expression of genes such as endogenous *BRCA1*. As such, I hypothesized that miSFITs could be applied to achieve precise, stepwise control over CAR expression levels (Figure 3.1).

To this end, I adapted Michaels and colleagues' previously reported miSFIT library responsive to microRNA-17 (miR-17). miR-17 is an attractive target given it is well-characterised miRNA [244, 245, 339-40] with reported expression in multiple T cell subsets [339, 341-5, 393-7]. This most notably includes CD8<sup>+</sup> T cells [344, 396-7], a subset that is often attributed to the efficacy of cellular immunotherapies [398]. Indeed, when CAR-T cells were manufactured solely from this subset, they have been demonstrated effective target cell eradication [399, 400]. Promisingly, Michaels and colleagues used this miR-17 responsive miSFIT library to tune PD-1, programmed death protein 1, transgene expression within Jurkat T cells, a cell line used within T cell activation studies [248]. This provides compelling evidence of this specific miSFIT library being used to tune transgene expression within T cells. As such, I selected this library as a basis for my study.

I used this miR-17 targeting miSFIT library in synergy with the NY-ESO-1 targeting DN-28z chimeric antigen receptor (CAR) [30]. New York squamous cell carcinoma 1 antigen (NY-ESO-1) is a promising target for CARs due to its specific expression in numerous tumour types, such as ovarian cancer and metastatic melanoma. Further, it is a cancer testes antigen, a category of tumour antigens that are expressed in male germ cells but not in healthy adult tissues, enabling specific targeting of cancer cells. Due to the above qualities, interest in NY-ESO-1 targeting CARs has grown. As such, I selected NY-ESO-1 to be the focus of my studies.

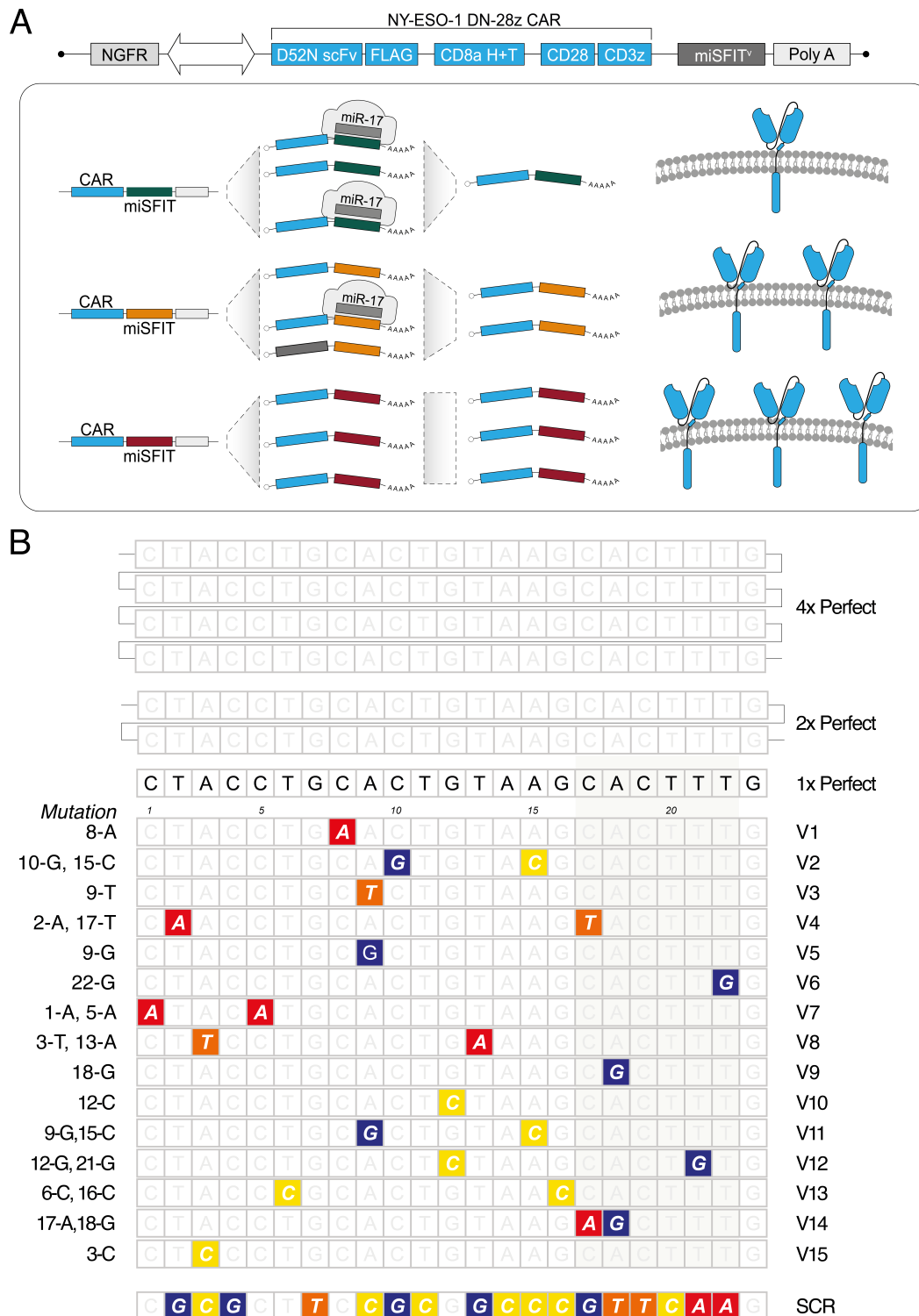
The NY-ESO-1 targeting CAR variant, DN-28z, is unique as it has been engineered by Maus and colleagues to enhance antigen binding and affinity [30]. More specifically, they mutated the D53 residue of the scFv from an aspartic acid (D) to an asparagine (N), resulting in DN. This resulted in the CAR demonstrating higher specificity to the NY-ESO-1 antigen presented on the HLA and with affinity on the micromolar range, akin to endogenous T cell receptor.

The DN-28z CAR's intracellular domain is composed of CD28, a co-stimulatory molecule, and CD3 $\zeta$ , an endogenous T-cell receptor activating and signalling domain. Its extracellular domain is composed of a FLAG epitope tag, used as a marker for antibody staining, and the D53N scFv.

I cloned the DN-28z CAR into a bi-cistronic expression vector library that encodes both a reporter gene, nerve growth factor receptor (NGFR) and a separate transcription unit where the CAR is placed under control of one of miR-17 miSFIT variants of my library (Figure 3.1). This expression vector library was housed in a lentiviral backbone, making it compatible with lentiviral packaging strategies.

Included within this library are 15 previously validated single and di-nucleotide variants (Figure 3.1). As established in my introduction, Michaels and colleagues demonstrated these MRE variants (referred to as V<sub>1</sub>, V<sub>2</sub>, ..., V<sub>15</sub>) to differentially recruit miR-17. This thereby changed the level of recruitment of RISC, resulting in different expression levels [219].

In addition, I included a perfectly complementary MRE to miR-17 (Figure 3.1). I expected this perfect target site to recapitulate the silencing behaviour observed in endogenous miR-17:MRE interactions due to its ability to perfectly Watson and Crick pair with miR-17. Because of this tuning, I would expect silencing of the DN-28z CAR. As such, this perfectly complementary MRE, referred to



**Figure 3.1: A system for tuning CAR expression** **A)** Core sequence of interest of DN-28z CAR-miSFITs expression vector levels. All vectors have a lentiviral-packaging-compatible backbone (not depicted). **B)** miSFIT sequences noting single/di-nucleotide point mutation and naming nomenclature used.

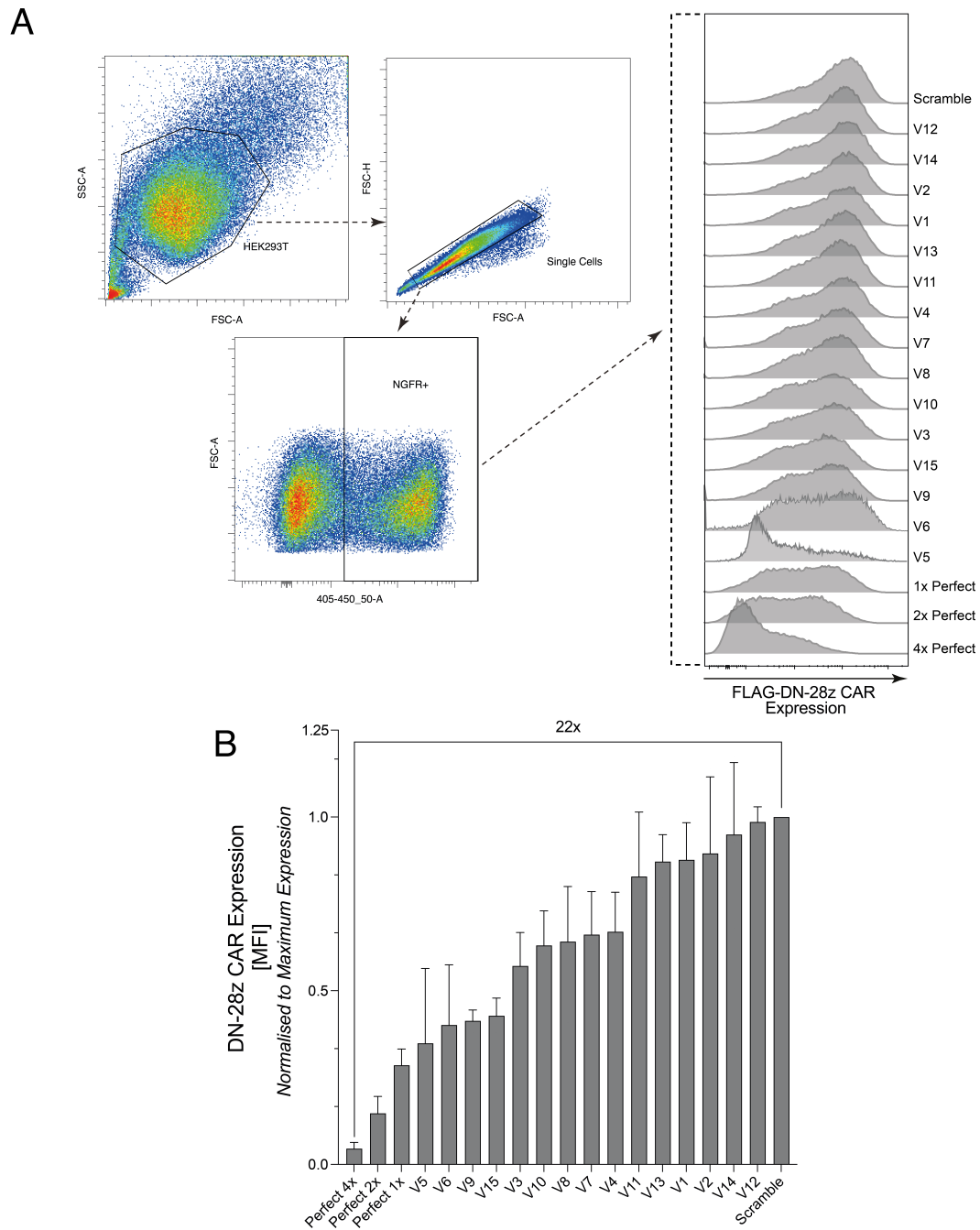
as 1x Perfect, served as a highly repressive control. In addition, I included two further repressive MRE variants that were duplicate and quadruplicate sequences of the 1x Perfect MRE, known as 2x Perfect and 4x Perfect (Figure 3.1). I would expect these miSFITs to ensure the silencing of DN-28z CAR due to having multiple cognate sequences to miR-17. This will result in more sites for miR-17 to bind with perfect complementarity, allowing for increased recruitment of the RISC and, ultimately, increased gene silencing [219]. Altogether, these variants increase the dynamic range of CAR expression to tune by providing increasing silencing strength.

I also include a Scrambled MRE variant (Figure 3.1). This variant bears low complementarity to miR-17 (6 of 23 nucleotides being complementary) and, therefore, I expected very low to no recruitment of the RISC. Therefore, I expect this variant to result in the highest level of CAR expression, providing a positive control.

For a proof-of-concept test, I transiently transfected this library of 19 different vectors into HEK293T cells [246, 247]. I measured DN-28z CAR expression by flow cytometry, staining for the FLAG epitope tag included within the DN-28z CAR extracellular domain. Assessing flow cytometry histograms of FLAG-DN-28z CAR expression demonstrated shifting populations of CAR expression (Figure 3.2a).

Normalised, average expression values of each miSFIT variant confirmed these observations (Figure 3.2b). More importantly, they demonstrated precise, stepwise levels of expression. In line with expectations, 4x Perfect, 2x Perfect, and 1x Perfect resulted in the lowest CAR expression level of all miSFITs, at 0.05, 0.15, and 0.29 MFI, respectively. Scrambled MRE resulted in the highest expression of the CAR, at 1 MFI. Intermediately, miSFITs demonstrated precise, stepwise levels of increasing expression. Comparing the lowest to the highest level of CAR expression demonstrated a fold change of 22x, providing a dynamic range in CAR expression. Taken together, these results provide convincing evidence that the miSFITs technology can precisely tune CAR expression.

While these results were promising, I conducted these tests in the HEK293T cell line. HEK293T is an immortalised human cell line derived from human embryonic kidney cells [246, 247]. I used them in my proof-of-concept tests due to their high permissibility to transient transfection [246, 247].



**Figure 3.2: miSFITs tune DN-28z CAR expression in HEK293T cells. A) Left: gating strategy used for assessment of DN-28z CAR expression in HEK293T cells Right: Representative flow cytometry histograms of DN-28z CAR expression (X axis, FLAG-DN28z CAR) in HEK293T cells. B) Average expression of DN-28z CAR across miSFITs in HEK293T cells. Fold change in surface expression is calculated by dividing the highest expression level by the lowest expression level, resulting in a 22x fold change. N=3 biological replicates, error bars report standard deviation.**



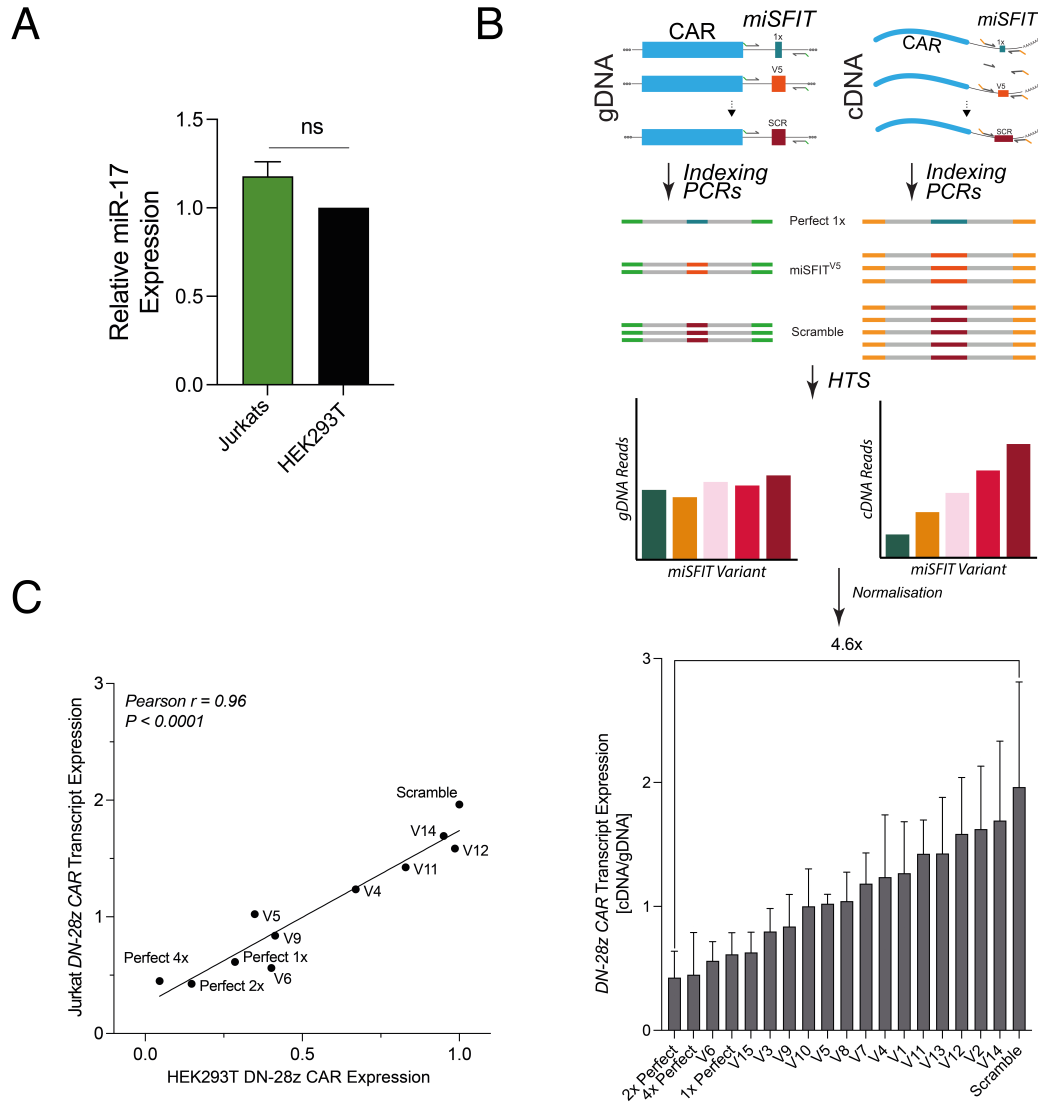
However, HEK293 T cells are not a representative cell type to study the effects of tuned CAR expression on T cell responses. As such, I sought to translate the DN-28z CAR-miSFITs system into a Jurkat T-cell line, an immortalised T cell line, routinely used to model T-cell biology and activation [248].

### 3.2 DEVELOPING A T CELL MODEL

Before introducing my DN-28z CAR-miSFITs library into Jurkat T cells, I sought to confirm miR-17 was present in Jurkat T cells given its key role in the miSFITs tuning system. To do so, I used real-time quantitative PCR with TaqMan probes to quantify expression levels of miR-17 in Jurkat T and HEK293 T cells, where I first demonstrated tuning. I additionally quantified RNU6B, RNA U6 small nuclear 6 B, expression to serve as an endogenous reference for normalisation. This assay revealed similar relative expression levels of miR-17 between the two cell lines. Jurkat T cells demonstrated a 1.18 relative miR-17 expression, while HEK293 T cells demonstrated 1 relative miR-17 expression (Figure 3.3a). This confirmed the expression of miR-17 in Jurkat T cells.

Having validated miR-17 presence in Jurkat T cells, I next focused on translating the DN-28z CAR miSFITs system into Jurkat T cells. I first sought to demonstrate transcript tuning of the DN-28z CAR. To do so, I employed high-throughput sequencing (HTS). In this experiment, I conducted a bulk, lentiviral transduction of Jurkat T cells with my entire library of DN-28z CAR-miSFITs. 72 hours post-transduction, I extracted the entire cell population's genomic DNA (gDNA) and RNA. After reverse transcribing the RNA into complementary DNA (cDNA), I then used primers specific to the 3' UTR of DN-28z CAR transgene, targeting the miSFIT sequence, to create PCR-amplicon libraries from the bulk-transduced Jurkat's gDNA and cDNA. I then subjected these libraries to HTS. After sequencing, I developed an algorithm that counted the number of times each miSFIT sequence appeared in gDNA and cDNA libraries, respectively. I calculated the transcript expression of each miSFIT by dividing the number cDNA counts by gDNA counts to normalise for integration rate.

Sequencing of the DN-28z CAR transcript demonstrated robust, stepwise-tuned expression across the miSFIT variants (Figure 3.3b). Indeed, Scrambled miSFIT demonstrated the highest CAR expres-



**Figure 3.3: Tuning DN-28z CAR transcript in T cell models.** **A)** RT-qPCR of relative miR-17 expression in HEK293T and Jurkat T cell line. RNU6b miRNA expression was used as a reference for normalisation. Error bars depict standard deviation. N = 3 biological replicates. **B)** Top: Schematic depicting strategy to measure DN-28z CAR transcript expression using high-throughput sequencing. Bottom: Average, normalised DN-28z CAR transcript expression calculated by dividing respective miSFIT's total RNA counts (cDNA) divided by total DNA counts (gDNA) assessed by high-throughput sequencing. Fold change in transcript expression is calculated by dividing the highest expression level by the lowest expression level, resulting in 4.6x fold change. N=3 biological replicates, error bars depict standard deviation. **C)** Pearson's correlation analysis between CAR expression observed in HEK293T and in RNA transcript expression of Jurkat T cells.  $r$  refers to Pearson's correlation coefficient and  $P$  refers to the  $P$  value of correlation analysis.

sion at 1.96 transcript expression, while 2x, 4x, and 1x perfect complementary miSFITs demonstrated the lowest CAR expression (0.43, 0.45, and 0.61 transcript expression, respectively). Once again, intermediate miSFITs demonstrated robust, stepwise levels of CAR expression. Comparing the miSFITs of the highest and lowest DN-28z transcript expression revealed an overall fold change of 4.6x. This excitingly demonstrates the robust tuning of DN-28z transcript expression in Jurkat T cells.

I questioned whether these results were consistent with my observations in HEK293T cells. To this end, I conducted Pearson's correlation analysis of each miSFITs' average CAR expression value between HEK293T and Jurkat. This revealed a very strong and significant correlation between the two cell types:  $\rho = 0.96$ ,  $P < 0.0001$  (Figure 3.3c).

As my ultimate goal was to tune the DN-28z CAR protein, I next focused my efforts on creating DN-28z CAR-miSFIT cell lines. I began by creating a subset of my DN-28z CAR-miSFITs library to transduce into Jurkat T cells. I selected miSFIT variants that would provide a representative picture of my CAR expression levels based on my observations in HEK293T and Jurkat experiments. I included 1x, 2x, and 4x Perfect complementary MREs as they tuned CAR expression to its lowest levels. I included Scramble MRE as it gave rise to the highest level of CAR expression. Therefore, these miSFITs provide the dynamic range of CAR expression. Of V1-15 miSFITs, I included: V4, V5, V6, V9, V11, V12, and V14 given their stepwise levels of CAR expression.

I lentivirally transduced this panel of DN-28zCAR-miSFITs into Jurkat T cells to create 11 polyclonal cell lines with stable CAR expression. I conducted this transduction optimising for single integrations (Figure 3.4a). I decided to do so to best preserve the tuning of CAR expression, as multiple integrations would lead to multiple copies of my vector being introduced, confounding expression levels.

I validated the transduction of my cell lines by extracting genomic DNA from each cell line, and PCR-amplifying the 3' UTR of the DN-28z CAR-miSFITs transgene, using primers specific to the template. I then purified this amplicon by gel electrophoresis and sent it for Sanger Sequencing (Figure 3.4b). Sanger Sequencing confirmed the genomic integration of each transduced miSFIT (Figure 3.4b).

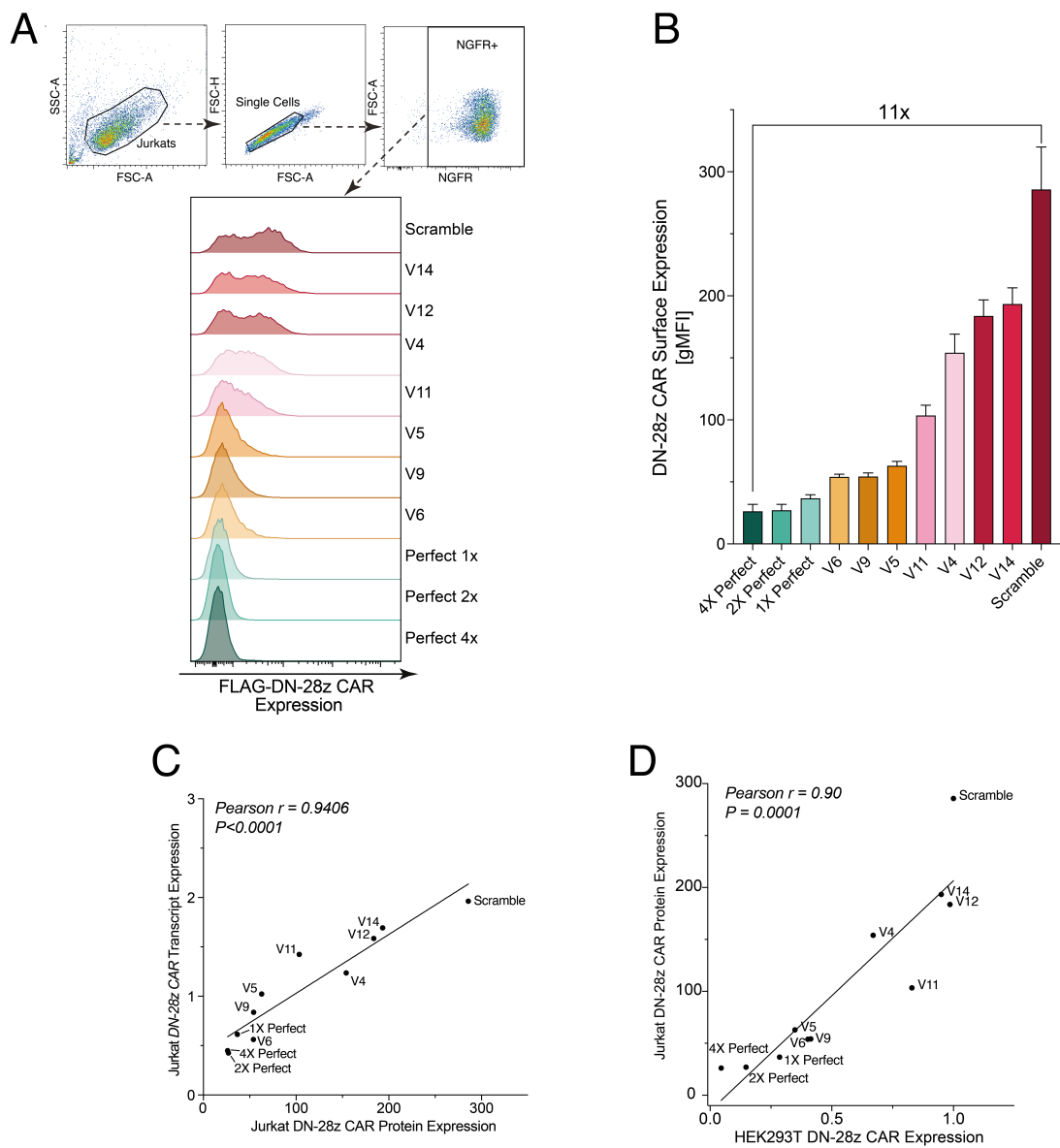


I then evaluated the expression of the CAR in each of these cell lines, using an anti-FLAG antibody and flow cytometry. Excitingly, miSFITs conferred stepwise control over expression levels with high reproducibility (Figure 3.5). Indeed, flow cytometry plots once again portrayed shifting populations of CAR expression (Figure 3.5a). This is further confirmed when evaluating the average CAR expression of each miSFIT cell line (Figure 3.5b). Consistent with my observations in HEK293T, 1X, 2X, and 4X perfectly complementary MREs yielded the lowest levels of CAR expression, as expected. Intermediate miSFITs variants conferred increasing levels of CAR expression in a stepwise fashion. Scramble MRE demonstrated the highest level of DN-28z CAR expression, as previously demonstrated. Furthermore, the miSFIT panel provided a broad, 11-fold dynamic range between the highest and lowest level of DN-28z CAR expression.

I questioned whether my observations in tuned CAR surface expression aligned with my observations in tuned CAR transcript expression. To this end, I conducted Pearson's correlation analysis of the miSFITs transduced between their measured CAR surface expression and RNA expression (3.5c). This revealed a very strong and significant correlation between CAR protein and RNA expression:  $\rho = 0.9406$ ,  $P < 0.0001$ . This implies that CAR expression is tuned similarly between RNA transcript and surface protein.

I further questioned whether these protein results were also consistent with my observations in HEK293T cells. To this end, I conducted Pearson's correlation analysis of each miSFITs' average CAR expression value between HEK293T and Jurkat. This revealed a very strong and significant correlation between the two cell types:  $\rho = 0.9$ ,  $P = 0.0001$  (Figure 3.5d). Thus, the tuning of CAR expression observed in my tests in HEK293T translates to my T cell model. Taken altogether, this data provides further evidence that the miSFIT technology is portable across cell types with little variation in the relative expression levels of each miSFIT variant.

Taken altogether, these results provide robust evidence of the precise tuning of CAR expression in my developed T cell model, answering the first question of my DPhil. Having done so, I moved on to my next question: what impact tuned CAR expression has on CAR T cell responses to their target antigen, more specifically, on T cell activation.



**Figure 3.5: miSFITs robustly tune CAR expression in T cell models.** **A)** Gating strategy and representative flow cytometry histograms of tuned DN-28z CAR expression (X-axis, FLAG-DN-28z CAR) in Jurkat T cell lines. **B)** Average CAR expression of each DN-28z CAR miSFIT cell line. N=3 biological replicates, error bars depict standard deviation. Fold change in surface expression is calculated by dividing the highest expression level by the lowest expression level, resulting in 11x fold change. **C)** Pearson's correlation analysis between Jurkat T cell model CAR surface protein and transcript expression. **D)** Pearson's correlation analysis between HEK293T and Jurkat T cell model CAR surface protein expression. *r* refers to Pearson's correlation coefficient and *P* refers to the *P* value of correlation analysis.

### 3.3 TUNING CAR EXPRESSION CORRESPONDINGLY TUNES EXPRESSION OF A T CELL ACTIVATION MARKER

The Jurkat T cell line I transduced the panel of DN-28z CAR-miSFITs is an ideal platform to address the second question of my DPhil. This cell line contains an NF- $\kappa$ B reporter. This reporter contains minimal promoters coupled to response elements for NF- $\kappa$ B which drive the expression of EGFP as a reporter for activity [220]. NF- $\kappa$ B is a family of transcription factors whose signalling cascades have multiple roles in T cell activation, such as the upregulation of early T-cell activation markers [249-255]. Thus, in this reporter system, after antigen engagement-elicited activation, endogenous NF- $\kappa$ B transcription factor complexes will bind to the NF- $\kappa$ B binding domains upstream of the minimal promoters. This, in turn, will result in the expression of EGFP as a direct readout. Thus, when activated by TCR-like stimulation, this Jurkat cell line will express EGFP, therefore, reporting the activation levels of NF- $\kappa$ B signalling and act as a model for T cell activation.

I leveraged this system to understand the effects of tuned CAR expression on T-cell activation. CAR T-cell activation initiates after a CAR engages with its specified antigen, presented on target cells. To simulate this, I co-cultured the developed DN-28z CAR-miSFIT cell lines with an artificial antigen presentation system. In this system, the target cell was a Chinese Hamster Ovary-K1 (CHO-K1) [256] cell line, engineered to express human CD58 (hCD58) and HLA-A\*02, human leukocyte antigen A\*02. HLA-A\*02 is an MHC-I (major histocompatibility complex I) molecule that binds peptide fragments and displays them on the cell surface for recognition by T cells. As discussed before, the DN-28z CAR is an MHC-restricted CAR that recognises the antigen NY-ESO-1<sub>157-165</sub> presented on HLA-A2\*02 [30]. Thus, CHO-K1 cells expressing HLA-A\*02 can be pulsed with the NY-ESO-1<sub>157-165</sub> peptide, presenting the antigen for NY-ESO-1-targeting DN-28z CAR-T cells to recognise, bind to, and activate in response. hCD58's expression strengthens the CAR: HLA-A\*02:NY-ESO-1<sub>157-165</sub> interaction between the CAR-T and the CHO-K1 cell by binding to the T-cell's CD2 antigen, a surface protein present on the T cell surface [392-4]. Thus, I used this synthetic antigen-engagement system as a vehicle to understand the effects of tuned CAR expression on T cell activation.

Using this synthetic activation system, I pulsed CHO-K1+hCD58+HLA\*AO2 cells with titrated concentrations of 9V NY-ESO-1<sub>157-165</sub> peptide. The 9V NY-ESO-1<sub>157-165</sub> peptide is a variant in which the cysteine (C) of amino acid 165 was substituted for valine (V), resulting in increased immunogenicity by enhanced binding affinity to the HLA-A\*02 [365]. I then co-cultured my DN-28z CAR-miSFIT cell lines for 24 hours. I then used flow cytometry to assess the CAR-T cell's EGFP levels as a readout for NF- $\kappa$ B activity and, more importantly, T cell activation.

Excitingly, flow cytometry plots of EGFP expression of the highest NY-ESO-1<sub>157-165</sub> concentration demonstrated cell populations that shifted across increasing CAR expression (Figure 3.6a). Seeking to quantify the magnitude of activation tuned, I calculated the median fluorescence intensity (MFI) of EGFP. Heatmaps depicting the average MFI of EGFP across miSFITs demonstrated tuned EGFP expression levels (Figure 3.6b). This demonstrates tuning of the magnitude of EGFP expression, and, therefore, the tuning of the magnitude of NF- $\kappa$ B and T cell activation.

To better visualise the tuning of EGFP expression, I fitted a log-logistic model, also known as the Hill function, to the average EGFP MFI of each DN-28z CAR-miSFIT cell line. This analysis similarly demonstrated distinct, tuned levels of expression across the different miSFITs (Figure 3.6c). The Scramble miSFIT (the burgundy, top line) demonstrated the highest EGFP expression. In contrast, 1x, 2x, and 4x Perfect miSFITs (bottom, turquoise-shaded lines) demonstrated the lowest levels of EGFP expression. Intermediate (middle, orange to pink lines) demonstrated stepwise levels of EGFP expression.

I sought to harness the information provided by the log-logistic model and sought to use it to make further inferences on tuned CAR expression's influence on T cell activation. To this end, I visualised the fitted highest activation value (EMax) of each miSFIT. 4x, 2x, and 1x Perfect miSFITs portrayed the lowest EMax values of 1415, 2083, and 2278. miSFITs V6, V5, V9, V11, V14, and V12 modelled with increasing, sequential Emax values. Scramble miSFIT had the highest EMax value at 63.3. Comparing Scramble and 4x Perfect EMax values, the highest versus the lowest, reveals a fold change of 4.7. Thus, this analysis portrayed stepwise levels, further validating tuned T cell activation (Figure 3.6d).



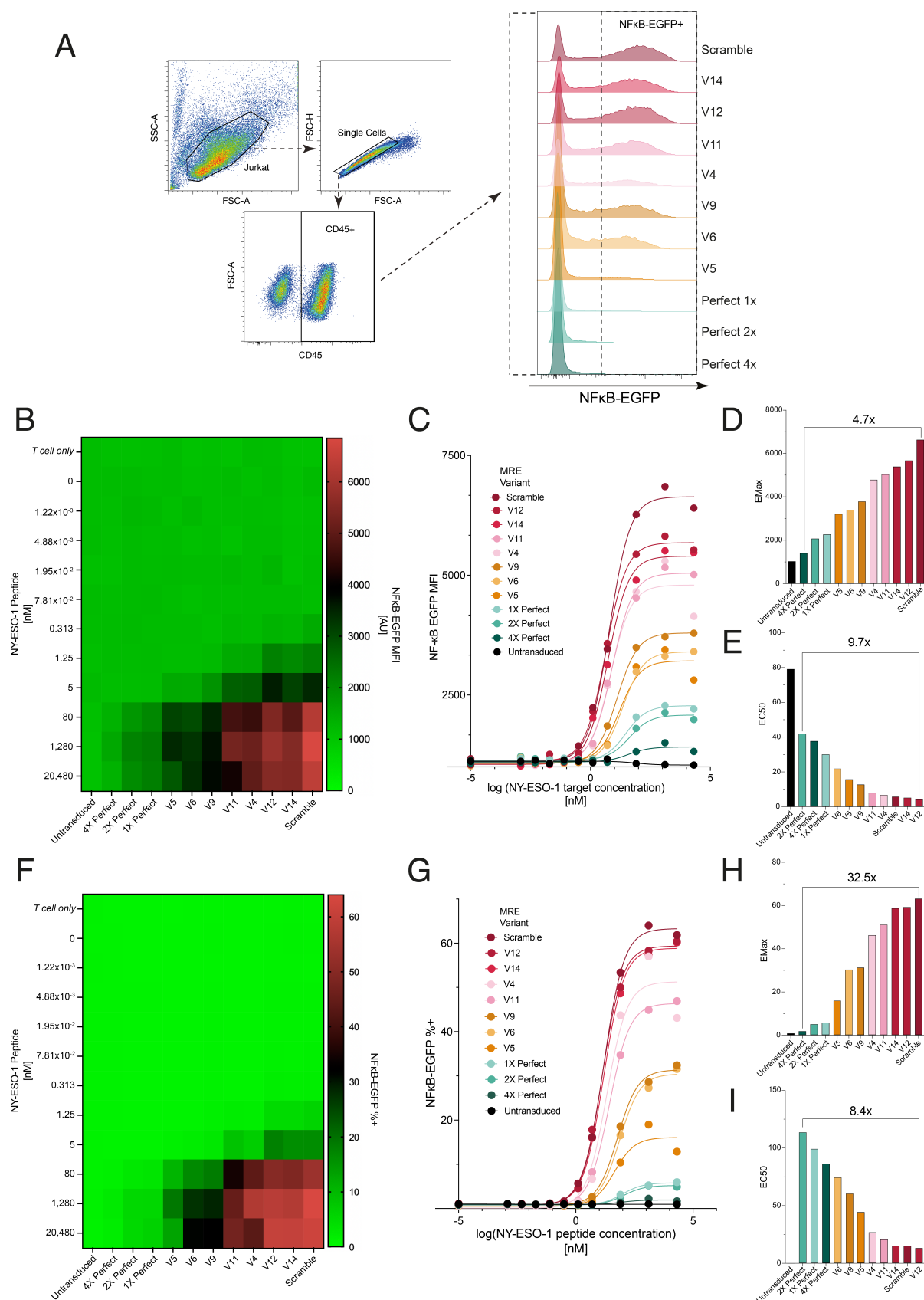


Figure 3.6: Tuned CAR expression correspondingly tunes a proxy for T cell activation. *Continued on the following page.*

**Figure 3.6: Tuned CAR expression correspondingly tunes a proxy for T cell activation.** *Continued from the following page.* **A)** Gating strategy (Left) and representative flow cytometry histograms (Right) of tuned DN-28z CAR expression (X-axis, NFκB-EGFP) in Jurkat T cell lines. **B)** Heatmap of NFκB-EGFP average median fluorescence intensity (MFI) across DN-28z CAR-miSFIT cell lines against pulsed concentrations of NY-ESO-1 9V peptide. N=3 biological replicates. **C)** 3-parameter log-logistic fitting of NF-κB-EGFP average MFI across miSFITs. Each coloured line represents a different miSFIT cell line. **D, E)** Bar plots of NFκB-EGFP average MFI 3-parameter log-logistic model maximum activation (EMax) and half maximal effective concentration (EC50) values of each miSFIT cell line. **D)** Fold change in EMax calculated by dividing highest by lowest EMax resulting in 4.7x fold change. **E)** Fold change in EC50 calculated by dividing highest by lowest EC50 resulting in 9.7x fold change. **F-I)** Repeated analysis as above using average NFκB-EGFP+ %+. **H)** Fold change in EMax calculated by dividing highest by lowest EMax resulting in 32.5x fold change. **I)** Fold change in EC50 calculated by dividing lowest by highest EC50 resulting in 8.4x fold change. **B&F)** For heatmaps, *T cell only* indicates negative controls featuring T cells without target cells or peptide

EC<sub>50</sub> is known as the half-maximal effective concentration. For my investigations, it is the concentration of antigen that provokes a response halfway between minimum and maximum T cell activation. Using my model, I considered the effects of tuned CAR expression on NF-κB-EGFP EC<sub>50</sub>. To this end, I plotted model-fitted EC<sub>50</sub> values for each DN-28z CAR-miSFIT cell line (Figure 3.6e). This analysis revealed decreasing stepwise values across miSFITs of increasing expression. 2x, 4x, and 1x Perfect miSFITs portrayed the highest EC<sub>50</sub> values of 42.05, 37.79, and 30.21. miSFITs V6, V5, V9, V11, V14, and V12 modelled with decreasing, sequential EC<sub>50</sub> values. Scramble miSFIT demonstrated the EC<sub>50</sub> value at 6.163. Comparing Scramble miSFIT's EC<sub>50</sub> with 2x perfect's EC<sub>50</sub>, the highest and lowest values, respectively, revealed a fold change of 9.7 (Figure 3.6e). This suggests that by tuning CAR expression, the amount of antigen CAR-T cells need to be exposed to before they activate is also tuned. Thus, this suggests that tuning CAR expression is an avenue to endow CAR-Ts with the ability to distinguish between different levels of NY-ESO-1 expression. This would be especially helpful in ensuring CAR Ts can discern between healthy and cancer cells. Taken altogether, these data provide compelling evidence of tuning of the magnitude of NF-κB-EGFP expression and, therefore, tuning of the magnitude T cell activation.

I asked whether my observations in tuned magnitude of NF-κB-EGFP expression extended to the proportion of cells that become activated. Visualising the average percentage positive for NF-κB-EGFP revealed similarly tuned levels (Figure 3.6f). Furthermore, reconducting the same log-logistic regression fitting on the average percent positive for each miSFITs portrayed tuned percentages, con-

sistent with my expression observations (Figure 3.6g). This demonstrates that tuning CAR activation not only tunes the magnitude of activation but also tunes the percentage of cells activated in response to antigen.

Extracting the EMax values of the model fit of the percent activated similarly revealed sequential, increasing levels (Figure 3.6h). 4x Perfect once again portrayed the lowest EMax value at 1.95, while Scramble portrayed the highest value at 63.3. Taken together, this demonstrated a fold change of 32.5 (Figure 3.6h). This further confirms that tuning CAR expression tunes the proportion of cells activated.

Model-fitted EC<sub>50</sub> values of the percentage of NF- $\kappa$ B-EGFP positive cells echoed my observations in the magnitude of NF- $\kappa$ B-EGFP, once again exhibiting decreasing EC<sub>50</sub> across miSFITs (Figure 3.6I). 2x, 1x, and 4x Perfect miSFITs once again portrayed the highest EC<sub>50</sub> values at 113.8, 99.4, and 86.54. The lowest EC<sub>50</sub> value was observed by the V12 miSFIT at 13.41. Comparing 2x Perfect's EC<sub>50</sub> value against V12's yielded a fold change of 8.4 (Figure 3.6I). This demonstrates how tuned CAR expression correspondingly tunes the percentage of NF- $\kappa$ B-EGFP positive cells. Intriguingly, it portrays how tuning CAR expression is an avenue to control how many cells activate in response to antigen. More significantly, it further illustrates how tuned CAR expression can be harnessed to endow CAR Ts with the ability to differentiate between antigen levels.

Taken together these results robustly illustrate that tuned CAR expression impacts T cell activation, providing an answer to the second question guiding my DPhil. Furthermore, it exemplifies how CAR expression is a strategy to control and direct T cell activation. I use this data to next characterise and visualise the relationship CAR expression has with T cell activation.

### 3.4 MODELLING T CELL ACTIVATION

The second question of my DPhil seeks to characterise CAR expression's influence on T cell activation and transcriptome. A method to facilitate this is fitting mathematical models to visualise this influence, as seen in one light in my magnitude and percentage analyses in NF- $\kappa$ B-EGFP. This can be

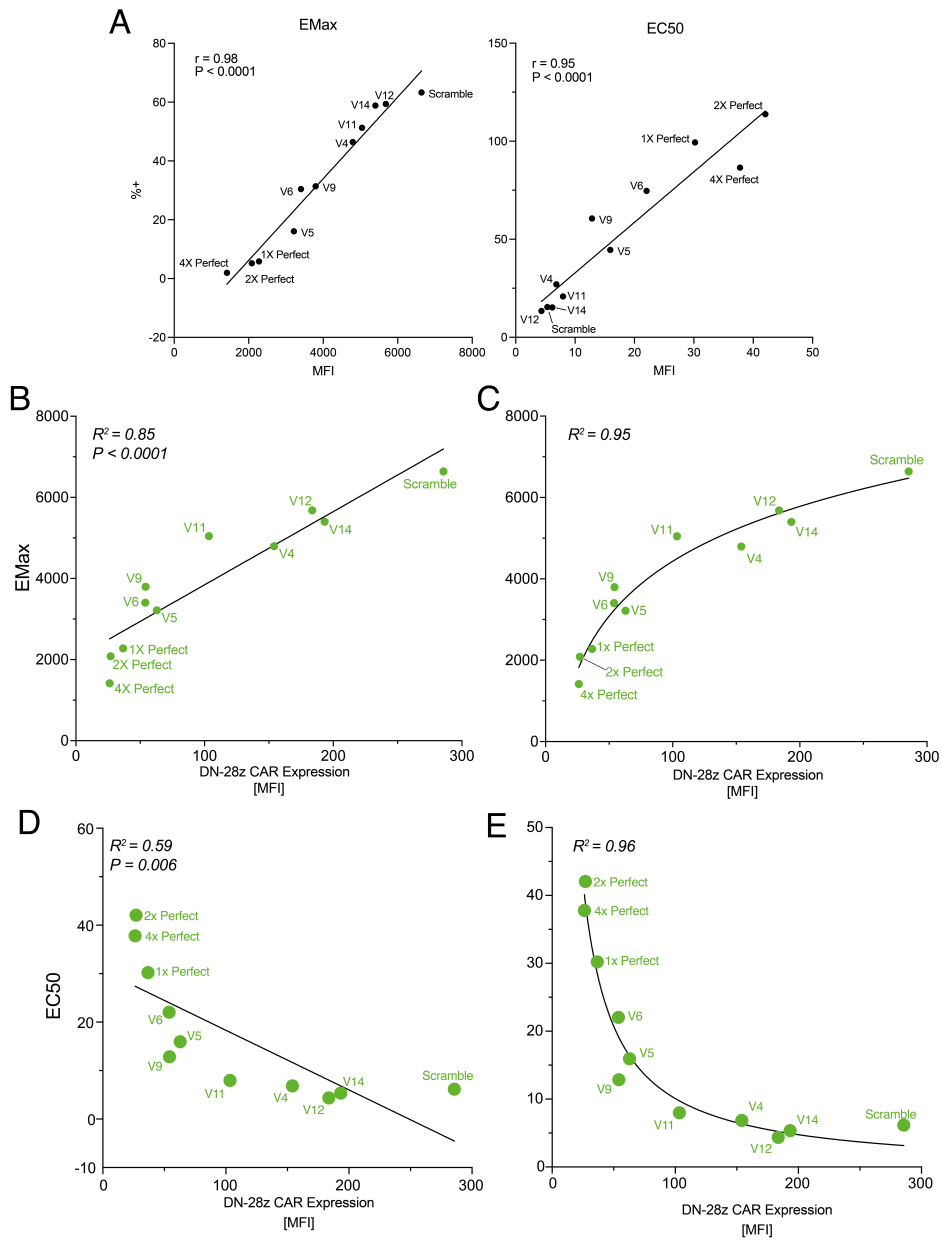
taken further to more directly dissect and characterise the relationship between T cell activation and CAR expression. To do so, I asked mathematical models best explain tuned CAR expression's impact on T cell activation.

To focus my efforts, I asked whether my observations on tuned activation translate between the magnitude of activation and the percentage of cells positive. To this end, I conducted Pearson's correlations analysis between the MFI and % positive of log-logistic model extracted EMax and EC<sub>50</sub> values (Figure 3.7a). This revealed very strong and significant correlation values for both EMax and EC<sub>50</sub> ( $r = 0.98$ ,  $P < 0.0001$  for EMax and  $r = 0.95$ ,  $P < 0.0001$  for EC<sub>50</sub>). Thus, I confirmed that my observations on NF- $\kappa$ B-EGFP translate between the magnitude of activation and the percentage of cells activated. Given this confirmation, I decided to focus my modelling efforts solely on the magnitude of activation.

To begin mathematical modelling, I took the model-fitted, highest activation NF- $\kappa$ B-EGFP expression (EMax) and average CAR expression for each miSFIT and conducted linear and logarithmic regression fitting (Figure 3.7b,c). Linear regression portrayed strong fitting ( $R^2 = 0.85$ ,  $P < 0.0001$ ) but did not capture numerous points, including many of the low CAR expression miSFITs (Figure 3.7a).

Logarithmic regression demonstrated very strong fitting ( $R^2 = 0.95$ ) (Figure 3.7b) and captured the majority of points. This highly suggests a logarithmic relationship best describes that between CAR expression and NF- $\kappa$ B-EGFP. This suggests that as CAR expression increases, NF- $\kappa$ B-EGFP activity rapidly increased but slows after a specific level. This implies that tuning CAR expression is a strategy to control NF- $\kappa$ B-EGFP activity and, therefore, T cell activity. This analysis highlights the utility of mathematical modelling in understanding the impact CAR expression has on the T cell activation.

In my EC<sub>50</sub> investigations, I observed data that suggested tuning CAR expression tunes the antigen requirement for CAR-T cell activation. I sought to formalise these observations by identifying a mathematical model that could explain this relationship. To this end, I took the model-fitted EC<sub>50</sub> values and average CAR expression for each miSFIT and conducted linear fitting (Figure 3.7d). This resulted in a poor fitting,  $R^2 = 0.59$ ,  $P = 0.006$ , and failed to capture the majority of points (Figure 3.7d).



**Figure 3.7: Mathematical modelling suggests tuning CAR expression controls T cell activation.** **A)** Pearson's correlation analysis between median fluorescence intensity (MFI) and % NF-κB-EGFP+ for model-fitted highest NF-κB-EGFP activation (EMax) and half maximal effective concentration (EC50) values.  $r$  refers to Pearson's correlation coefficient and  $P$  refers to the  $P$  value of correlation analysis. **(B, C)** Linear (B) and Logarithmic (C) regression analysis between average CAR expression and EMax for each miSFIT. **D, E)** Linear (D) and Logarithmic (E) regression analysis between average CAR expression and EC50 for each miSFIT.  $R^2$  and  $P$  refer to the goodness of model fit parameters.

However, linear regression fitting sheds light on alternative, potential mathematical models. This is due to how it failed to capture  $V_6$ ,  $V_5$ ,  $V_9$ ,  $V_{11}$ ,  $V_4$ ,  $V_{12}$ , and  $V_{14}$ . Together, these appeared to form a valley-esque shape, leading to me hypothesizing that these might be better captured by an inverse relationship. Furthermore, my observations implied that increasing CAR expression reduced the  $EC_{50}$  value for each DN-28z miSFIT cell line. This would be very well explained by an inverse model. Indeed, inverse regression fitting confirmed this hypothesis. It portrayed a very strong and significant fitting,  $R^2 = 0.96$ , capturing the majority of points very well, including the highlighted miSFITs. Notably, miSFITs  $V_5$ ,  $V_9$ , and  $V_{11}$  formed the inflection point of the curve. This suggests that these miSFITs' CAR expression levels represent the minimum expression level to rapidly induce T cell activation. In essence, this demonstrates that CAR expression tunes the  $EC_{50}$  value in an inverse manner. This, therefore, implies that CAR expression's influence on the antigen requirement is best explained by an inverse relationship.

Overall, my mathematical model analyses suggest how CAR expression tunes T cell activation.

### 3.5 DISCUSSION

My doctoral work is guided by two main questions:

1. Is it possible to tune CAR expression to precise levels of expression utilizing the miSFITs tuning system?
2. If so, how do CAR expression levels impact T cell activation?

In this subchapter, I provided compelling evidence to answer these core questions. I demonstrated the successful deployment of the miSFITs tuning system to tune CAR expression. I then established a T cell model to validate the robust, stepwise tuning of CAR expression. These results provided an answer to my first question. I then used this model to demonstrate the tuning of NF- $\kappa$ B-EGFP expression and, therefore, T cell activation. This provided a compelling answer to my second question. Finally, I leverage these observations in a mathematical model to infer how CAR expression can be a potential method to control T cell activation.

This work focused on the NY-ESO-1 targeting DN-28z CAR. I selected this CAR for two reasons. The first: the growing interest in NY-ESO-1, due to its specificity to cancer cells, and the engineered variant that displayed TCR-line affinity and specificity for the antigen. However, as discussed in my introduction, many CARs have been developed (e.g. notably CD19-4-1BBz and CD19-28z, the variants approved for FDA use [257]), while novel CARs are being developed constantly. And while my results robustly demonstrate that adapting a validated miSFTs library did not need further optimisation, it is likely that the validation I conducted will need to occur for different NY-ESO-1 targeting CAR variants. Note that this extends to CARs of different architectures and targeting different antigens [21].

For my experiments in understanding the effects of tuned CAR expression on activation, I limited my investigations to a single marker to assess activation. Even more, it was a synthetic activation reporter. While my results were promising and robust, they do not accurately capture the endogenous activation markers observed within primary cells. Ideally, being able to assess a cell surface marker associated with activation, such as CD69, would be a more appropriate strategy to assess the impact on T cell activation.

Additionally, I limited my focus on the impact of tuned CAR expression on the T cell transcriptome to T cell activation primarily due to logistical limitations. However, T cells are multifunctional cells, with well-demarcated lineages and functions. While my results on a tuned T cell activation marker are very promising, they are also limited. Assessing how CAR expression impacts other genes associated with T cell activation and function would be the next progression.

For mathematical modelling, I utilised only two models: linear and logarithmic. This is primarily due to ease of application and interpretation. Furthermore, preliminary data visualisation indicated what models may best fit the data. However, for future markers and genes assessed, it would be beneficial to consider further models, given that not all relationships will be fitted or explained by linear or logarithmic models.

Overall, these results heavily suggest tuning CAR expression impacts the T cell activation. However, they were demonstrated in a T cell model, Jurkat T cells. This is a major limitation as Jurkats

are an immortalised cell line that do not recapitulate all the functionality of primary T cells. Indeed, recent findings by Felce et al. support this, where they demonstrate that transcriptomic expression only represents a minority of those in endogenous, primary T cells, suggesting that Jurkats represent a highly simplified model of T cells [391]. Thus, it would be more appropriate to translate my DN-28z CAR-miSFITs system into primary T cells to have a more accurate, comprehensive picture.

Taken altogether, my investigations indicated that I had promising results, and potential answers, for the questions guiding my DPhil. However, moving to primary cells will be an exciting opportunity to not only confirm these observations but also expand what insights I can make on tuned CAR expression.



# 4

## Tuning CAR expression in primary T cells

In the previous subchapter, I established a T cell model in which I successfully tuned CAR and saw its effects on T cell activation. In this chapter, I translate my preliminary findings to donor-derived human T cells. This is a key step as donor-derived T cells are the cells in which CAR-T cell therapies are generated and infused into patients. Thus, any findings related to tuned CAR expression observed would likely translate to these therapies. Furthermore, these observations capture a more representative picture of tuned CAR expression on T cell activation, as these tests were in human primary cells rather than a simplified T-cell model.

In this subchapter, I demonstrate the successful tuning of CAR expression in human primary CD8<sup>+</sup> T cells. I then show tuned expression of CD69, an early T cell activation marker, thus demonstrating tuned T cell activation. I use these models to suggest potential relationships between this activation marker and use these results to suggest how CAR expression impacts its role in T cell activation. These findings portray how tuned CAR expression could help better design CAR control strategies.

#### 4.1 TRANSLATING miSFITs INTO DONOR-DERIVED, HUMAN T CELLS

To begin my investigations in human, donor-derived T cells, I first selected a further subset of miSFITs used to tune CAR expression. This is due to logistical reasons, as I was limited in the number of blood donors to extract T cells from and by the number of T cells required for lentiviral transduction. I based my selection on my observations in the Jurkat T cell model. Akin to my previous transductions, I selected miSFITs to best illustrate the range of CAR expression. I included 4x Perfect MRE to represent the lower bound of expression. On the opposing end, I included Scrambled MRE to represent the highest level of expression. To select intermediate miSFITs that would give rise to representative, stepwise levels of CAR expression, I calculated the expression range defined by 4x Perfect and Scrambled MREs. I then took this value and divided it by the total number of miSFITs I wanted

| <i>Calculated, ideal expression</i> | <i>miSFIT with expression closest to value</i> |
|-------------------------------------|------------------------------------------------|
| 26                                  | Perfect 4x (26)                                |
| 91                                  | V <sub>11</sub> (103)                          |
| 156                                 | V <sub>4</sub> (154)                           |
| 221                                 | V <sub>14</sub> (193)                          |
| 286                                 | Scramble (286)                                 |

**Table 4.1:** Selecting miSFITs to transduce into primary T cells

to transduce, 5. These calculations are shown below.

$$Scramble_{expression} - Perfect4x_{expression} = Expression\ Range$$

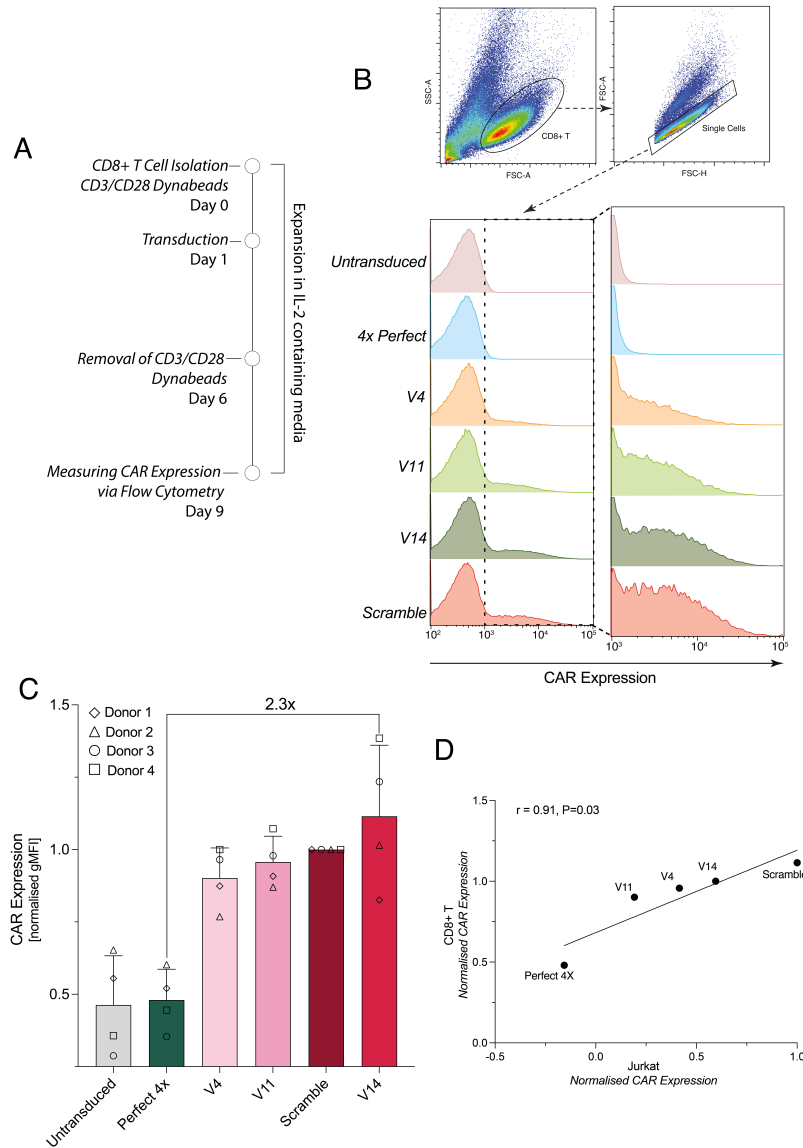
$$286_{gMFI} - 26_{gMFI} = 260_{gMFI}$$

$$\frac{Expression\ Range}{5\ miSFIT\ lines} = Step\ in\ expression\ between\ each\ line$$

$$\frac{260}{5} = 65\ expression\ step$$

Each step was calculated to be 65 gMFI between. The starting level of expression, Perfect 4x, was 26 gMFI. Thus, the idealised expression steps were: 26 (Perfect 4x), 91, 156, 221, and 286 (Scrambled). Referring to my observations in Jurkat T cell models, I selected miSFITs whose expression were closest to these calculated levels, as tabulated below: Thus, I included V<sub>11</sub>, V<sub>4</sub>, and V<sub>14</sub> miSFITs with Scrambled and Perfect 4x miSFITs to be transduced into CD8<sup>+</sup> T cells.

I transduced this panel of miSFITs in CD8<sup>+</sup> T cells. In brief, CD8<sup>+</sup> T cells were first extracted from blood cones attained from 4 donors and then placed in IL-2-containing media with anti-CD3/CD28 Dynabeads. These culturing conditions simulate the activation cues for T cell expansion. The next day, T cells were transduced with lentivirus. Once again, I optimised my transductions for single integrations to prevent multiple integrations, confounding the tuning of CAR expression. The T cells were left to expand in IL-2-containing media for 10 days before assessing CAR expression via 9V-tetramer antibody, an antibody specific to the DN-28z CAR (Figure 4.1a).



**Figure 4.1: miSFITs robustly tune CAR expression to stepwise levels.** **A)** Overview of generation of human, primary CD8<sup>+</sup> T DN-28z CAR miSFIT lines. **B)** Flow cytometry gating strategy and histogram plots of CAR Expression across DN-28z CAR miSFITs. **C)** Normalised, average geometric median fluorescence intensity (gMFI) of CAR expression of DN-28z-miSFIT CD8<sup>+</sup> T cell lines. CAR expression was normalised on a donor basis by dividing the expression of samples by respective Scramble control. 2.3x fold change of CAR expression between DN-28z-miSFIT lines. Diamonds represent Donor 1, triangles Donor 2, circles Donor 3, and squares Donor 4. **D)** Pearson's correlation analysis of normalised, average CAR expression between CD8<sup>+</sup> 28z-miSFIT cell lines and Jurkat cell lines. CD8<sup>+</sup> T cell CAR expression was normalised on a donor basis by dividing the expression of samples to donor-respective Scramble miSFIT. Jurkat CAR expression was normalised on a biological replicate basis by dividing the expression of samples to respective Scramble control. Pearson's  $\rho$  and P values represent correlation parameters.

Excitingly, histograms of CAR expression demonstrate populations shifting towards higher levels (Figure 4.1b). This is accompanied by increasing numbers of cells of higher expression. Average, geometric median fluorescence intensity (gMFI), normalised on a donor-basis by dividing samples' expression by respective Scramble control, of each miSFIT shows robust, stepwise levels of increasing CAR expression (Figure 4.1c). T cells transduced with Perfect 4x MRE demonstrated the lowest level of CAR expression at 0.48. This is slightly greater than background levels of expression at 0.47, as seen in untransduced cells. V<sub>4</sub> and V<sub>11</sub> demonstrated stepwise levels of expression, at 0.9 and 0.95, respectively. Interestingly, V<sub>14</sub> demonstrated a higher expression at 1.1 than Scramble MRE's expression of 1. This is likely explained by the notable variation in tuning observed in V<sub>14</sub>.

I asked to what degree the tuning observed in donor T cells aligned with my T cell model. To this end, I conducted Pearson's correlation analysis of CAR expression per miSFIT between my observations in Jurkat T cell models and in donor-derived CD8<sup>+</sup> T cells (Figure 4.1d). This analysis reveals a very strong correlation,  $r = 0.91$ ,  $P = 0.03$ . This suggests miSFITs tune CAR expression as robustly and reliably in human donor T cells as they did in the T cell model. This reaffirms the applicability of the miSFIT technology across different cell types.

Taken altogether these results provide clear evidence of precise tuning of CAR expression to stepwise levels, answering the first question of my DPhil. I next sought to assess the effects of tuned CAR expression on T cell activation.

#### 4.2 TUNED CAR EXPRESSION TUNES AN EARLY T CELL ACTIVATION MARKER

In the previous subchapter, Jurkat T cell models expressing tuned CAR expression demonstrated tuning of NF- $\kappa$ B-EGFP, a marker for T cell activation. Having successfully tuned CAR expression in human, primary T cells, I asked what impact this had on tuned T cell activation.

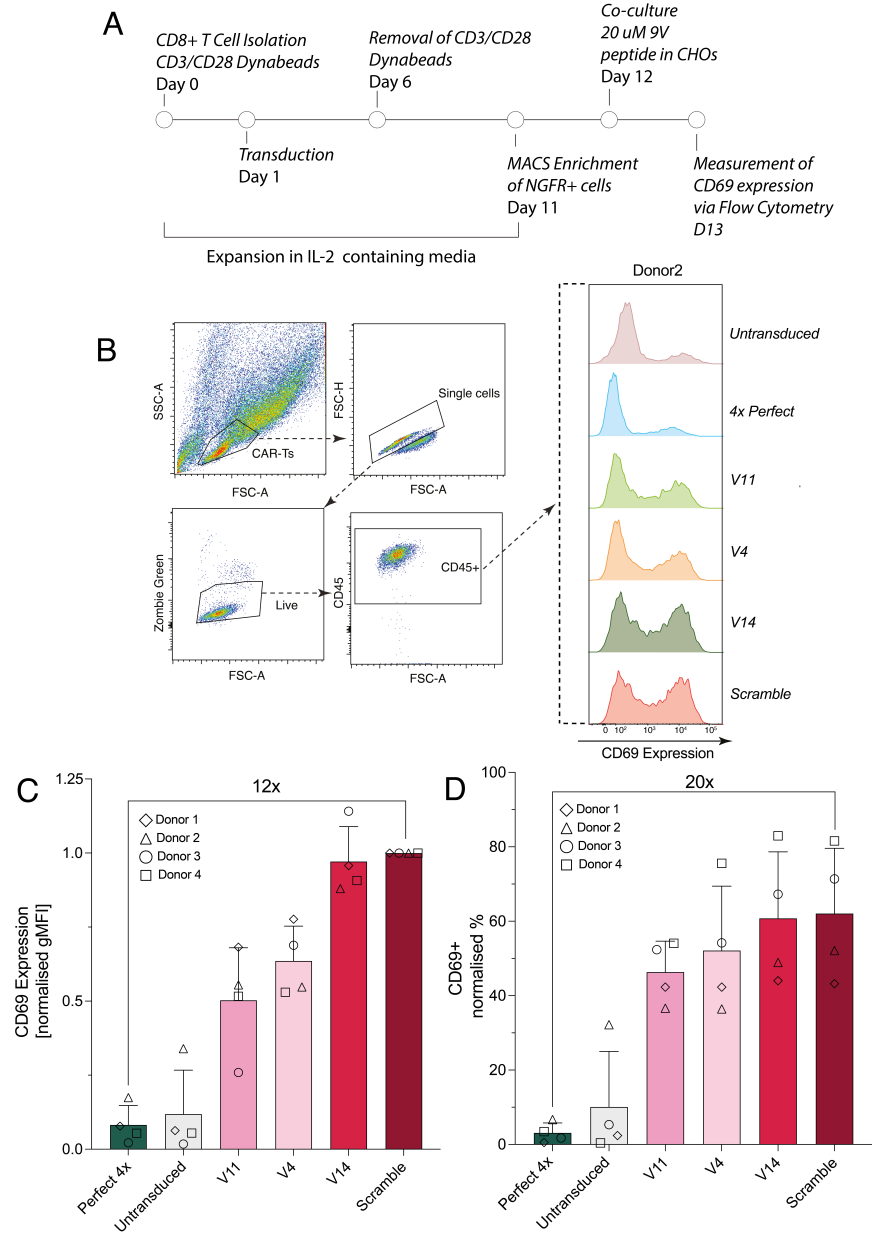
To this end, I simulated antigen-based T cell activation using the primary T cell lines I previously generated. In brief, after expanding transduced T cells for 10 days, I subjected them to magnetic-activated cell sorting. This was to enrich the transduced T cells by targeting my transduction marker,

nerve growth factor receptor (NGFR). To do so, I used antibodies against NGFR, conjugated with magnetic beads. After an overnight rest, the T cells were then activated by using my optimised synthetic activation system. In essence, I co-cultured my enriched T cells with CHO-K1 target cells, pulsed with NY-ESO-1<sub>157–165</sub>, for 24 hours. Due to limited cell numbers, I pulsed two NY-ESO-1 peptide concentrations: 0 $\mu$ M and 20 $\mu$ M. I included 0 $\mu$ M to assess background activation in my T cells. I included 20 $\mu$ M to ensure maximum T cell activation. This concentration is based on what was required for maximum activation levels in Jurkat T cell models.

After the 24-hour co-culture, I used anti-CD69 antibodies and flow cytometry to assess CD69 expression levels (Figure 4.2a). CD69 is a T cell activation marker that is swiftly upregulated in response to T cell antigen binding. It has been implicated in regulating cytokine release and the homing and migration of activated T cells [258-261]. CD69's roles in controlling key T cell activation responses make it an ideal target to assess the tuning of T cell activation. Furthermore, as I previously discussed, CD69 is an endogenous T cell marker, thus is a more direct, representative readout of T cell state.

Remarkably, histograms of CD69 expression demonstrate populations of increasing CD69 expression across miSFITs of increasing CAR expression (Figure 4.2b). I sought to quantify the tuned magnitude of activation. I calculated the average gMFI of CD69 expression of each miSFIT cell line, normalised for each respective donor. Excitingly, this revealed precise, stepwise levels of expression (Figure 4.2c). Indeed, Perfect 4x demonstrated the lowest level of expression at 0.12 normalised gMFI. Scramble miSFIT, once again, portrayed the highest level of expression at 1 normalised gMFI. miSFITs V11, V4, and V14 demonstrated intermediate, stepwise levels of expression. Comparing Scrambled MRE and Perfect 4x's average expression reveals an expression fold change of 12x.

I asked if tuned CAR expression also tunes the proportion of cells activated. I calculated the mean percentage of cells positive for CD69 expression. Excitingly, this analysis similarly demonstrated stepwise levels of percentage cells activated (Figure 4.2d). Scramble miSFIT exhibited the highest average proportion of cells activated at 62%. V14, V4, and V11 demonstrated decreasing proportions of 61%, 52%, and 46%, respectively. Perfect 4x exhibited the smallest average proportion of activated cells at 3%. Comparing the proportion of activated between Scrambled and Perfect 4x demonstrates a range



**Figure 4.2: CAR expression correspondingly tunes CD69 to stepwise levels. A)** Overview of protocol for CD69 expression assessment in tuned DN-28z CAR miSFIT CD8+ T cell lines. **B)** Flow cytometry gating strategy and histograms for CD69 expression across DN-28z CAR miSFIT CD8+ T cell lines. **C)** Normalised, average geometric median fluorescence intensity (gMFI) of CD69 expression of DN-28z-miSFIT CD8+ T cell lines. CD69 expression was normalised on a donor basis by dividing expression of samples by respective Scramble control. 12x fold change of CD69 expression between DN-28z-miSFIT lines. Diamonds represent Donor 1, triangles Donor 2, circles Donor 3, and squares Donor 4. **D)** Normalised, average percent positive of CD69 expression of DN-28z CAR miSFIT CD8+ T cell lines. Normalised on a donor basis by subtracting donor's percent of unstimulated untransduced CD69 %+ from donor's respective DN-28z CAR miSFIT lines. 20x fold change of CD69 %+ between DN-28z-miSFIT lines. Diamonds represent Donor 1, triangles Donor 2, circles Donor 3, and squares Donor 4.

of 59% and fold change of 20x.

Taken altogether, these results clearly demonstrate the effects of tuned CAR expression on T cell activation, answering the second question of my DPhil.

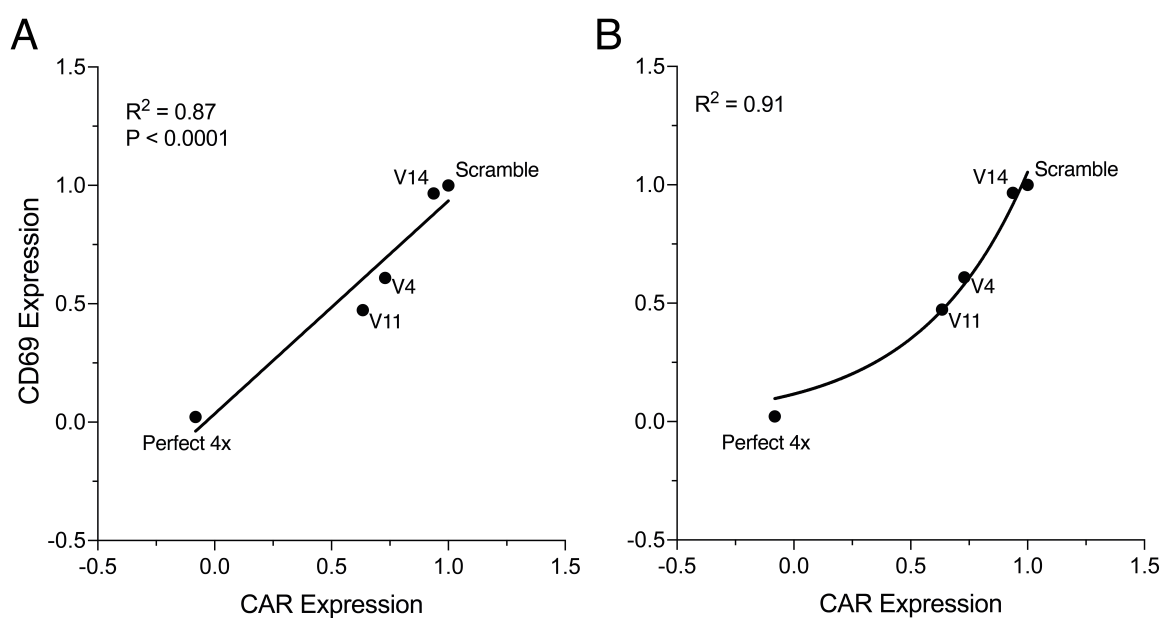
I next asked what mathematical model best describes the relationship between tuned CAR and CD69 expression. To this end, I took the average, donor-normalised gMFIs of both CAR expression and CD69 expression and conducted linear regression fitting (Figure 4.3a). This revealed strong fitting:  $R^2 = 0.87$ ,  $P < 0.0001$ . However, miSFITs V<sub>11</sub> and V<sub>4</sub> were not captured well by the linear model. Thus, I considered other mathematical models that could better explain the relationship. Interestingly, an exponential growth model better fit the data and captured V<sub>4</sub> and V<sub>11</sub>:  $R^2 = 0.91$  (Figure 4.3b). This suggests that increasing CAR expression induces a rapid increase in CD69 expression. Further, this indicates that tuning CAR expression is an avenue to control T cell activation. For example, CAR expression could be tuned with the V<sub>11</sub> miSFIT, tuning CD69 expression to an inflection point, giving rise to T cell activation before rapidly increasing. While these observations are promising, further points are needed to ensure these observations are bona fide.

### 4.3 DISCUSSION

Taken altogether, these results represent a major step forward in my DPhil project. I successfully demonstrated miSFITs robustly tuning CAR expression in human, donor-derived primary T cells. Furthermore, I demonstrated how tuned CAR expression accordingly tunes CD69, an activation marker with roles in T cell immune response. I then use this data to suggest that an exponential mathematical explains the relationship between CAR and CD69, indicating a rapid induction of this early T cell marker. These results definitively and clearly answer the questions motivating my DPhil.

Due to logistical constraints, I was only able to select 5 miSFITs to tune CAR expression with. Remarkably, I observed five, distinct levels of expression. However, a notable gap in expression can be observed between Perfect 4x and V<sub>4</sub>. Thus, while I observed stepwise levels of expression, they were not even steps, as I calculated. Possible explanations for this could be in how I calculated CAR





**Figure 4.3: Regression fitting reveals exponential relationship between CAR and CD69.** Regression analysis DN-28z CAR-miSFIT CD8+ T cell lines normalised, average geometric median fluorescence intensity (gMFI) of CAR and CD69 expression using linear (A) and exponential (B) mathematical models. CAR expression and CD69 expression values were normalised on a donor basis by dividing the expression of samples by respective Scramble miSFIT.  $R^2$  and associated P values refer to model fitting.

expression in my T cell model. I calculated CAR expression by the median fluorescence intensity of the FLAG-DN-28z CAR+ gating in my flow cytometry observations. However, this likely skewed CAR expression calculations to be higher than they actually are. Thus, my calculations to determine which miSFITs to transduce into primary T cells were likely skewed, leading to higher CAR expression miSFITs to be selected.

However, including additional miSFITs would address this gap. Being able to fill this gap in expression would provide further information in my downstream investigations in T cell activation tuning and in mathematical modelling. For example, having more levels of CAR expression would increase the number of tuned levels of CD69 expression. This would impact my mathematical modelling analysis. While an exponential relationship appeared to best fit 5 points, new points may cause this model to collapse. This would require a new relationship to be identified, as I did when originally considering the linear relationship. Alternatively, new points may further support the model. Thus, increasing the number of tuned levels of CAR expression within primary T cells is a further goal that would provide beneficial granularity to my studies.

CD69 is a key marker in T cell activation. However, it does not comprehensively cover the complexity of T cell activation. Indeed, cellular response and behaviour is a synergistic, cohesive effort between multiple genes and involves intricate signalling pathways. Being able to capture this granularity would be beneficial to my studies. For example, understanding how all the different components in T cell activation are impacted by tuned CAR expression could provide insights into how T cells activate at different levels of CAR expression. T cells may leverage one gene more than another at a lower level of CAR expression, or vice versa at higher levels of expression. Thus, having a comprehensive atlas of all genes and proteins involved in T cell activation against multiple levels of CAR expression could provide valuable insights.

In a similar vein, T cells are a multifunctional cell type, and activation is but one process involved in the immune response. It does not capture key T cell responses such as effector function and cytotoxicity, the ability to secrete cytokines (such as interleukins, interferons, and TNF family members) and cytotoxins (such as perforin, granzymes, and granulysin). Nor does provide information in mem-

ory differentiation or T cell regulation. Nor does it provide information, more relevantly, on T cell dysfunction or exhaustion, a T cell state leading to the loss of effector function and memory capacity. Thus, expanding the CAR expression atlas beyond T cell activation to include all genes and proteins involved in T cell response is an exciting and fruitful opportunity to aim for. A technology that I could use to address these opportunities is RNA-sequencing, a technology that captures the entirety of the transcriptome.

In this subchapter, I completed a key step within my DPhil. I successfully translated the miSFITs tuning system into primary T cells to robustly tune CAR expression. I further investigated its effects on CD69, a marker of T cell activation. These observations provide robust data that answer the questions guiding my DPhil. However, they provide just scratch the surface and could be greatly expanded using RNA-sequencing.

# 5

Assessing whole transcriptomic responses  
within human CD8<sup>+</sup> T cells expressing tuned  
chimeric antigen receptors

In the previous chapter, I successfully translated the miSFIT tuning system into human CD8<sup>+</sup> T cells, tuning both DN-28z CAR and CD69 surface expression, confirming my observations in Jurkat-based T-cell models.

In this chapter, I expand upon those findings and utilize the entire DN-28z CAR-miSFITS library in synergy with single-cell RNA-sequencing to create a transcriptomic atlas against CAR expression. I undertake a guided analysis, investigating specific cell markers to investigate CAR expression's impact on the T cell transcriptome. I leverage gene signatures related to activation, dysfunction, and memory, assessing the impact of CAR expression levels on these key T cell capacities. I use these data to reveal the relationship between CAR expression and T cell dysfunction and suggest how these findings may address an outstanding issue hamstringing the CAR-T field. I then investigate how CAR expression impacts T cell subset proportions. I conclude this chapter by performing an unbiased analysis to identify novel genes, regulons, and pathways that depend on CAR expression.

I previously demonstrated the tuning of DN-28z CAR surface expression into four distinct levels in human primary CD8<sup>+</sup> T cells. Furthermore, I demonstrated that this resulted in four distinct levels of surface CD69 expression. This provided convincing evidence that tuned CAR expression impacts T cell activation, but I wanted to expand upon these findings. More specifically, I sought to increase the resolution of my assessment, increasing the number of levels to tune expression well beyond 4. To accomplish this, I employed the entirety of my miSFIT library, allowing me to tune CAR expression to 19 levels.

Additionally, I sought to move beyond assessing just one marker, given how little information this provides and the limited number of inferences that could be made. In a similar vein, a single marker, by no means, comprehensively represents a single process within cells. For example, T-cell activation is a complex, multi-faceted, and tightly regulated process involving several genes and proteins, each playing various roles in signaling cascades [262, 263]. Being able to gain an insight into how some of these 'players' and the overall process are impacted by CAR expression would be beneficial for the chimeric antigen receptor field.

Investigating multiple genes at once would allow us to begin making these insights—well beyond

just T-cell activation. As such, I employed RNA-sequencing technology to assess the entire transcriptomic response to different levels of CAR expression. Single-cell RNA-sequencing has been a seminal advancement of RNA-sequencing technology due to its ability to resolve heterogeneity within a population of cells. For my questions, using it would allow me to directly relate CAR expression to the transcriptional state on a cell-by-cell basis.

As such, I transduced a bulk population of T cells with a pool of lentivirus containing all 19 variants of my miSFIT library. For this transduction, I optimized the dose of lentivirus to maximize a single integration per cell. Once again, this was to ensure my intended precise, tuning of CAR expression and avoid the confounding effects on expression due to multiple integrations.

A further benefit of using single-cell RNA sequencing technology is that it can capture the miSFIT sequence. The commercially available 10X Genomics Single Cell 3' gene expression method captures 3' end of RNA transcripts, the location of the miSFIT sequence within the DN-28z CAR-miSFIT transcript. As such, I can identify which miSFIT each cell is transduced, providing an alternative method to demultiplex.

I introduced the DN-28z CAR-miSFITs library into three donors to have biological replicates. After allowing the T cells to expand, I enriched my transduced cells via magnetic-activated cell sorting on a transduction marker, NGFR (nerve growth factor receptor), to control the proportion of untransduced cells within my samples. To accomplish this, I spiked in a proportion of untransduced cells to each donors' samples.

I simulated antigen-based activation by co-culturing the T cells for 24 hours using my previously described synthetic, CHO-K1 activation system. To prevent CHO-K1 transcript contamination in downstream RNA library preparation, I then fluorescence-activated cell sorted on blood cell lineage marker, CD45. I then created single-cell RNA-sequencing libraries using the 10X 3' platform from donor-pooled, FACS-sorted cells.

To avoid batch-to-batch sequencing variation, I pooled and loaded samples from all 3 T cell donors into a single cell capture reaction. To subsequently assign cells to individual donors, I used genetic demultiplexing. In brief, bulk RNA sequencing samples from each donor were used to identify unique

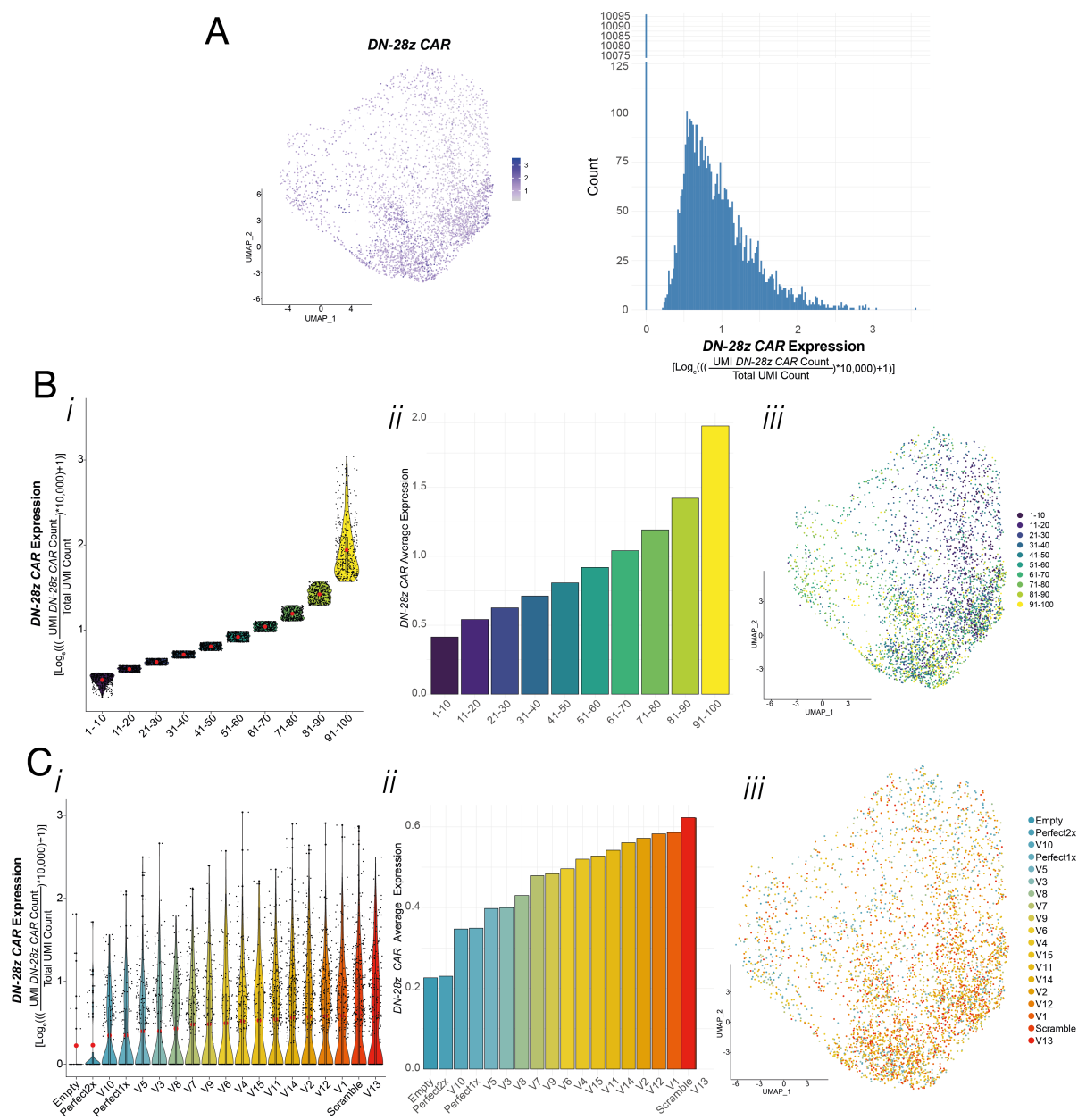
SNPs that subsequently allowed us to assign donor identities to our single cells. To harness the unique barcodes of miSFITs, I took cDNA created from 10X GEM reaction and created PCR-amplicon, targeting the miSFIT sequence in the 3' UTR of the DN-28z CAR transgene, based library (see Chapter 2.15 for a more detailed breakdown and visualized in Figure 2.1).

To assess expression levels of the CAR transcript, I created a custom reference genome, appending on the *DN-28z CAR-miSFIT* transgene onto the GRCh38.p13 genome assembly. I used this reference for 10X genomics' Cell Ranger pipeline, which performed read alignment, filtering, barcode counting, and UMI counting. From here, I conducted necessary quality control filtering, including the number of unique genes detected and the percentage of reads mapping to the mitochondrial, and demultiplexing via genetic SNPs and miSFIT barcode sequences, as detailed further in Chapter 2.15.

## 5.1 CONFIRMING TUNED DN-28z CAR EXPRESSION IN SINGLE-CELL RNA SEQUENCING

The first question of my DPhil asks whether it is possible to tune CAR expression to precise, stepwise levels. Thus, I first sought to validate the tuning of the *DN-28z CAR* transgene within my single-cell RNA sequencing library. After demultiplexing and quality control cell filtering, 13,921 single cells formed one large, primary entity on UMAP visualization (Figure 5.1a). *DN-28z CAR* transcripts were detected in 27% of all cells (3,825 of 13,921 cells) and formed a 'north-south polarity' on UMAP visualization, where higher *DN-28z CAR* expression concentrated in the southernmost area of the UMAP entity (Figure 5.1a), suggesting *DN-28z CAR* expression as a factor driving overall transcriptional differences.

To better understand CAR expression's impact on gene expression in downstream analysis, I took the 3,825 cells with detected expression and identified their percentile rank according to their *DN-28z CAR* expression level (Figure 5.1b). I then grouped this ranking into deciles to create ten CAR-expression-based bins, composed of approximately 380 cells each (Figure 5.1b). Both *DN-28z CAR* expression distribution and mean confirm stepwise levels of expression across the 10 bins (Figure 5.1b.i, ii). Cells binned to lower percentiles were located generally in the eastern section of the UMAP



**Figure 5.1: Confirming tuned CAR expression in single-cell RNA-sequencing.** **A)** Left: UMAP visualization portraying DN-28z CAR expression, Right: Distribution of CAR expression. **B)** i: Distribution of CAR expression per percentile rank, CAR expression bin ii: Average CAR expression per percentile rank, CAR expression bin iii: UMAP visualization portraying localization of cells in each percentile rank, CAR expression bin. **C)** i: Distribution of CAR expression per miSFIT ii: Average CAR expression per miSFIT iii: UMAP visualization portraying localization of cells demultiplexed to a miSFIT



entity. In concordance with *DN-28z CAR* expression, higher percentile binned cells shifted and concentrated in the southernmost area (Figure 5.1b.iii).

I next sought to assess CAR expression in miSFIT demultiplexed cells. 4,288 cells were demultiplexed to a miSFIT, 31% of the total number of cells (Figure 5.1c). When evaluating the distribution of *DN-28z CAR* expression for each miSFIT, a shift towards higher levels occurred when traversing from maximal tuning miSFITs (Perfect 2x and Perfect 1x) to no tuning as in the Scramble miSFIT (Figure 5.1c.i). Indeed, calculating the average of *DN-28z CAR* expression per miSFIT confirms stepwise levels of tuned expression (Figure 5.1c.ii). UMAP visualization of miSFIT demultiplexed cells reveals a shift in cell density in concordance with higher CAR expression levels (compare Figure 5.1a and Figure 5.1c.iii).

I wanted to confirm whether these observations were in line with those in Jurkat T cell models. To this end, I performed Pearson's correlation analysis comparing average *DN-28z CAR* expression observed for each miSFIT in this single-cell experiment versus Jurkat T cell RNA and protein expression (Figure 5.2a). This analysis confirmed that tuned CAR expression in this experiment strongly and significantly correlated with my T cell model observations (RNA  $\rho = 0.70$ ,  $P = 1.26 \times 10^{-3}$  and protein  $\rho = 0.80$ ,  $P = 5.35 \times 10^{-3}$ ) (Figure 5.2a).

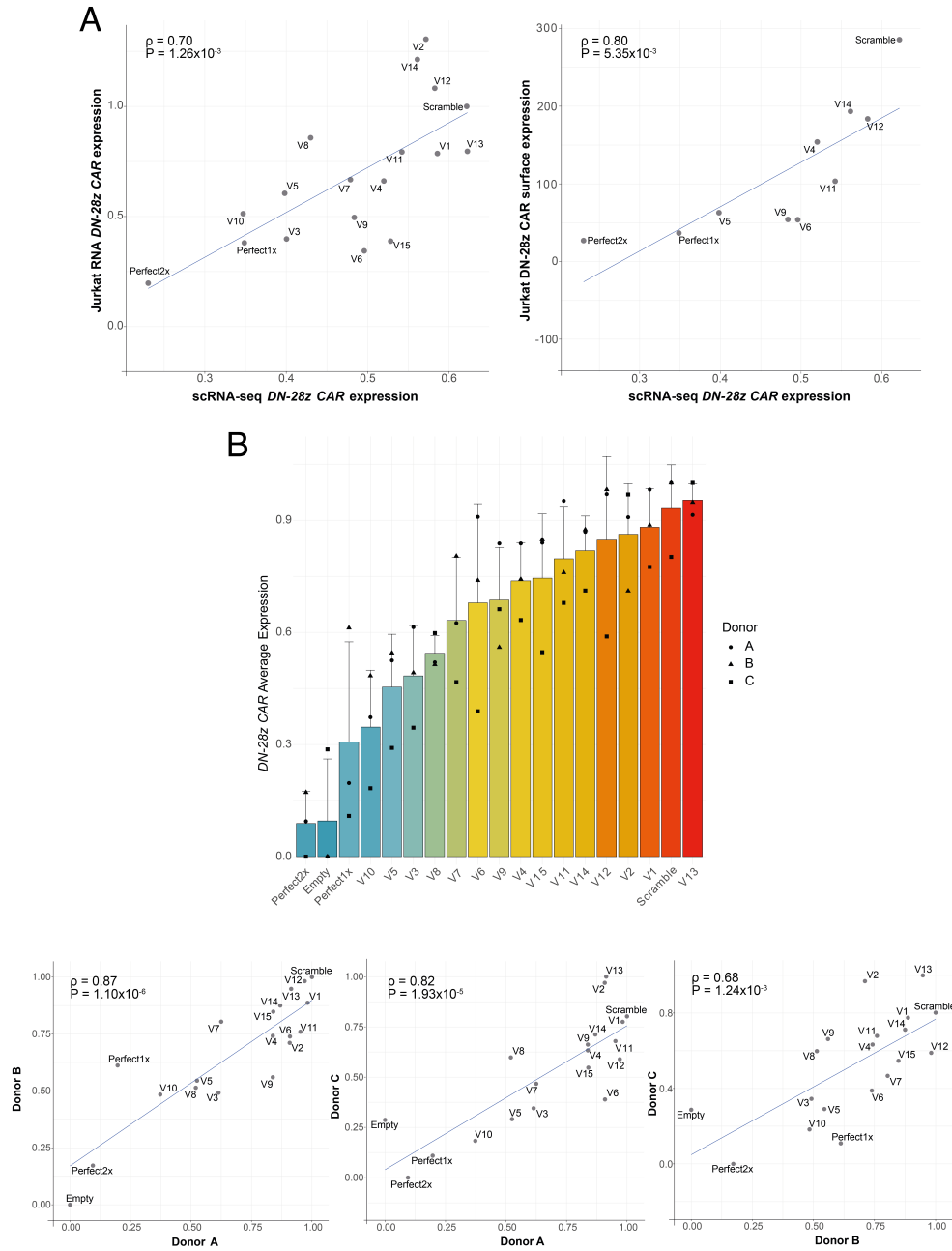
I next sought to confirm the tuning of *DN-28z CAR* expression across the constituent donors of my single-cell RNA sequencing experiment. Plotting the average expression of *DN-28z CAR* of each miSFIT on a donor basis confirmed stepwise levels expression (Figure 5.2b).

Finally, I asked whether the degree of tuning of the *DN-28z CAR* transcript by each miSFIT was similar between donors. Conducting Pearson's correlation analysis comparing *DN-28z CAR* expression of each miSFIT between the three donors revealed strong and significant correlations between all three donors (Figure 5.2b):

$$A \text{ vs } B: \rho = 0.87, P = 1.10 \times 10^{-6}$$

$$A \text{ vs } C: \rho = 0.82, P = 1.93 \times 10^{-5}$$

$$B \text{ vs } C: \rho = 0.68, P = 1.24 \times 10^{-3}$$



**Figure 5.2: miSFITs robustly tune CAR expression across cell types and donors. A)** Pearson's correlation analysis comparing miSFIT single-cell RNA sequencing *DN-28z* CAR expression and Jurkat RNA expression measured in high-throughput sequencing, left, and Jurkat T cell model surface protein expression measured in flow cytometry quantification, right. **B)** Average CAR expression per miSFIT on a donor basis (biological replicates  $N = 3$ ). Error bars depict standard deviation. **C)** Pearson's correlation analysis comparing miSFIT CAR expression between donors, A, B, and C. Pearson's correlation coefficient and P value provided.

Taken together, these results once again demonstrate the robust tuning of the *DN-28z CAR* transgene within my single-cell RNA sequencing experiment. Importantly, the developed binning strategies within my single-cell RNA-sequencing library lay the important foundation for my downstream analysis, allowing me to visualize and associate expression patterns of different genes to a continuum of CAR expression levels. These observations firmly address the first motivating question of my DPhil.

## 5.2 EFFECTS OF TUNED CAR EXPRESSION ON T CELL ACTIVATION, DYSFUNCTION, AND MEMORY

Having once again demonstrated the tuning of CAR expression, I sought to move on to address the second question of my DPhil: how does tuning CAR expression impact the T cell transcriptome? To guide this analysis, I looked towards the current literature within the CAR-T therapy field and identified key marker genes used to provide insights on different T cell functions. This included T cell activation, cytotoxicity, differentiation, and memory [181, 264-266].

T cell exhaustion, more recently called dysfunction, is a major concern and limitation related to T cell therapies. Numerous studies have provided preliminary evidence that CAR expression plays a significant role in the onset of T cell exhaustion but do not thoroughly assess the effects of different CAR expression levels. T cell dysfunction is associated with regulatory, now more commonly view as inhibitory receptors, PD-1, CTLA-4, and LAG3. Wanting to assess CAR expression's impact on dysfunction, I included the genes encoding these receptors, as well as other genes associated with T cell dysfunction, to have a first-pass insight. In later subchapters, I more directly address T cell dysfunction.

Many of the selected genes' expression patterns correlated with the expression of *DN-28z CAR*. UMAP visualizations portrayed co-localisation of *DN-28z CAR* of higher expression levels with all selected activation genes *CD69*, *PRF1*, and *IL2* [264, 267-273]] and exhaustion genes *LAG3*, *HAVCR2*, and *CTLA4* (Figure 5.3a). In contrast, memory genes *CCR7*, *IL7R*, and *SELL* demonstrated higher expression levels where *DN-28z CAR* expression was lower.

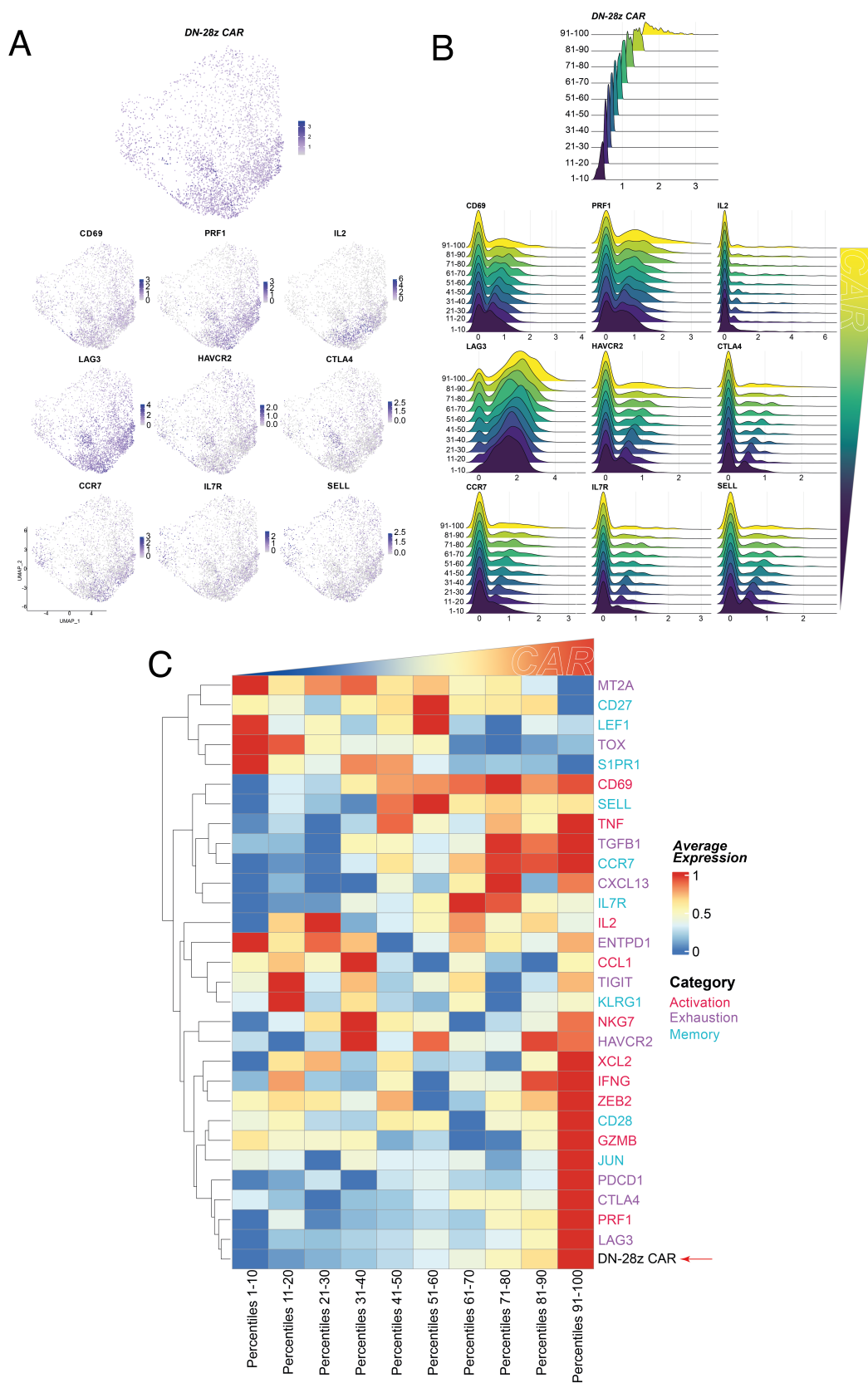


Figure 5.3: Effects of tuning CAR expression on the T cell transcriptome. *Continued on the following page.*

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**Figure 5.3: Effects of tuning CAR expression on the T cell transcriptome.** *Continued from the previous page.* **A)** UMAP visualizations depicting expression localization of cell markers representing T cell activation, dysfunction, and memory. **B)** Expression distribution of selected T cell markers across percentile rank, CAR expression bins. **C)** Hierarchically clustered heatmap depicting average expression of an expanded selection of T cell markers of percentile rank, CAR expression bins. Average expression was scaled for visualization purposes such that gene expression minima was set to zero and the maxima set to 1.

Indeed, this is confirmed when evaluating the expression distribution of these genes across increasing percentile rank bins, as a general shift towards high expression is observed for all genes, including my selected memory genes (Figure 5.3b).

To better understand the relationship different genes had with CAR expression, I took the cells identified in each CAR expression bin and calculated the average expression of the selected genes. I then used hierarchical clustering to group genes of similar expression and visualized it on a heatmap to identify trends and patterns (Figure 5.3c).

Excitingly, this analysis revealed varying levels of expression across the different CAR expression bins for all genes. *LAG3*; *CTLA4*, both exhaustion markers; and *PRF1*, encoding for cytotoxic protein perforin[274], all demonstrated patterns of similar tuned expression patterns to *DN-28z CAR* and clustered closest with it. These genes have a strong positive association with CAR expression.

Another cluster of genes, composed of effector genes *CD69* and *TNF*; dysfunction genes *TGFB1* [275-279] and *CXCL13* [280]; and memory genes *CCR7*, *IL7R*, and *SELL*, also demonstrated a positive relationship in their expression pattern across CAR expression bins (Figure 5.3c). Notably, both *IL7R* and *SELL* demonstrate a switch-like expression pattern, having low levels of expression until CAR expression bin 5 or 6, rapidly turning on and tapering off slightly to a high basal level of expression after peak expression (Figure 5.3c). This implies the expression of these genes could be harnessed by directing CAR expression to a specific level. Memory-associated genes *LEF1* and *S1PR1* portray a negative expression pattern against CAR expression. They demonstrate high expression levels in low CAR expression bins but significantly dropped after Percentiles 41-50. Interestingly, *TOX*, recently demonstrated to be expressed in exhausted T cells [175, 181] clustered with these memory genes and portrayed a strikingly similar expression pattern.

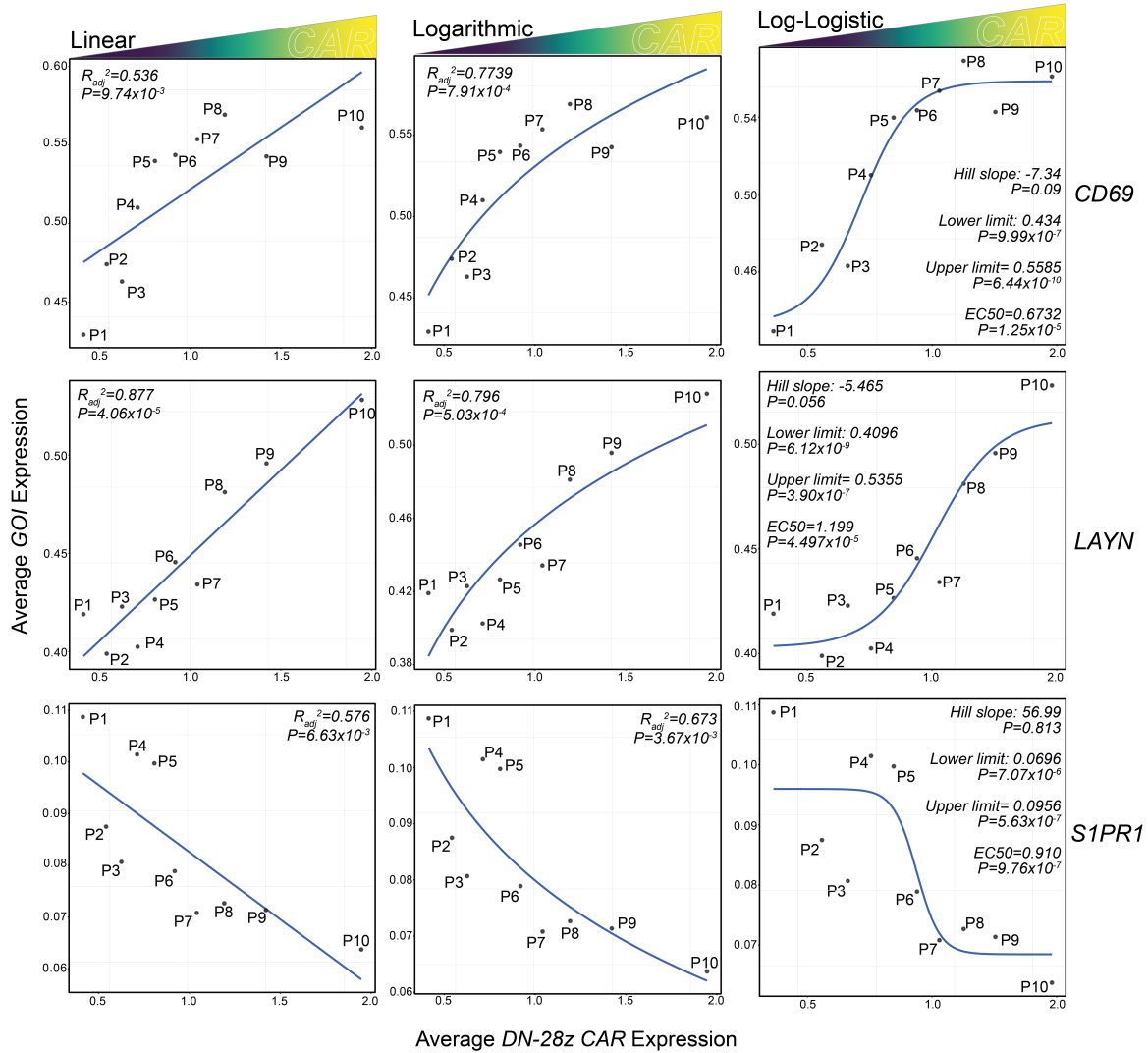
These findings firmly address the second question of my DPhil. More specifically, they clearly demonstrate that *DN-28z CAR* expression impacts the T cell transcriptome. This includes genes related to T cell activation, cytotoxicity, exhaustion, and memory. Importantly, these data provide indicative evidence of CAR expression influencing T cell dysfunction, activation, and memory.

### 5.3 MODELLING CAR EXPRESSION'S INFLUENCE ON T CELL MARKERS

These observations prompted me to explore the relationship these affected genes had with CAR expression. While the previous subchapter provided a preliminary insight into this (Figure 5.3c), I wanted a more direct assessment and visualization to guide my analysis. As I have done previously in chapters 3 and 4, I used regression fitting to visualize what mathematical model best explains the impact of CAR expression on these genes.

To this end, I took the average expression of a subset of T cell marker genes and plotted them against CAR expression, using the CAR expression bins. I then conducted linear, logarithmic, and log-logistic (also known as the Hill function) regression fitting (Figure 5.4). I selected T cell markers that represent major T cell phenomena, e.g. activation, exhaustion, and memory, in order to provide an initial insight on the relationship these had with CAR expression. My subset included T cell activation marker *CD69*[259], T cell repression marker *LAYN*[281], and T cell memory marker *S1PR1*[282, 283].

*CD69* and *LAYN* had positive relationships with *DN-28z CAR* expression (Figure 5.4). Regression analysis heavily suggested *CD69* and *LAYN* expression have logarithmic or sinusoidal relationships with CAR expression, capturing majority of CAR expression points. Interestingly, in log-logistic modelling, *CD69* demonstrated a steep increase in expression before leveling off after CAR expression bin P6, the inflection point in expression. This suggests a rapid induction of *CD69* at low CAR expression levels. This is in alignment with *CD69*'s role in early T cell activation [259]. *LAYN*, in contrast, portrayed a slight delay until CAR expression bin P4, before eliciting a rapid rise in expression. *LAYN* has been shown to have a role in regulating and suppressing T cell function [281].



**Figure 5.4: Characterising relationships between CAR expression and T cell function markers.** Linear, logarithmic, and log-logistic regression fitting of cell marker genes, *CD69*, *LAYN*, and *S1PR1* respectively representing T cell activation, dysfunction, and memory. Average expression per percentile rank, CAR expression bin was used for each gene in regression analysis. For linear and logarithmic regression analysis, adjusted  $R^2$  and P value reflects model fitting. For log-logistic regression analysis, 4-parameter log-logistic function was used and portrayed on plots is hill slope, lower bound, upper bound, and EC50 and corresponding P value from model fitting. P1, P2, ..., P10 correspondingly represent Percentile Rank 1-10, Percentile Rank 11-20, ..., and Percentile Rank 91-100.

Thus, these results suggest how *LAYN* regulatory capacities may be induced past specific levels of CAR expression. *S1PR1* portrayed a negative relationship with CAR expression. Majority of models demonstrated poor fitting, failing to capture the majority of points. However, a logarithmic model appeared to best explain the relationship between *S1PR1* and *DN-28z CAR*. This suggests a steep drop in *S1PR1* activity at early CAR expression levels (Figure 5.4).

In the previous subchapter, I investigated how tuned CAR expression impacted CD69 surface expression in human primary T cells. My analysis revealed an exponential relationship, in contrast to what I observed in this analysis. However, as raised previously, this is likely explained by the additional points of CAR expression providing more information for models. Thus, this analysis provides a more comprehensive portrait of the relationship of *CD69* and CAR expression.

This analysis highlights the utility of modelling relationships between CAR expression by offering a potential explanation of when specific genes may be induced or most active across CAR expression levels. Combined with my findings in 5.2 (Figure 5.3) these results shed light in how CAR expression influences the T cell transcriptome, thus providing a robust answer to the second question of my DPhil.

#### 5.4 CAR EXPRESSION CAN BE USED TO CONTROL T CELL DYSFUNCTION

In answering the main questions guiding my DPhil, I observed how CAR expression directly influences numerous genes on T cell dysfunction. As previously mentioned (Chapter 1.2), one of the most pressing challenges in CAR-T cell therapy is T cell exhaustion [2, 151-154, 166, 284-290], a state of dysfunction impacting several of the key functions. In this state, T cells have been associated with impaired proliferative ability, cytotoxicity, excessive differentiation, and upregulation of inhibitory receptors, such as PD1 and CTLA-4 [147, 148, 150]. This state has been demonstrated to limit CAR T therapy's successful translation in different cancers [18,44]. As such, a current, major focus within the field is to engineer and develop strategies to develop exhaustion-resistant CAR-T cells.

I previously observed *PDCD1*, *CTLA4*, and *LAG3*, which encode for inhibitory receptors with key



roles in T cell exhaustion [150, 373-376], are directly affected by tuned CAR expression, I hypothesized whether CAR expression levels control T cell exhaustion, a major limitation in the CAR-T field. This is characterised by reduced effector function and reduced cytokine production [147-148, 150, 156].

For a long-standing time, the T cell exhaustion and activation transcriptional states have been intertwined [369, 370]. This is likely due to regulatory mechanisms being simultaneously upregulated to fine-tune T cell activation and control it to prevent nonspecific activation and autoimmunity [370-372]. However, by comparing the transcriptional states of cells expressing different combinations of TIM3 and PD-1, key inhibitory receptors, Singer and colleagues successfully deconvolved T cell activation and dysfunction modules, while also reporting a naive/memory-like module. These gene signatures provide the perfect means to address whether CAR expression controls T cell exhaustion. Leveraging Singer and colleagues' findings, I calculated each gene signature mean enrichment score across my CAR expression bins and visualized the distribution in scores (Figure 5.5a). Genes from any signature that were undetected in cells were not included in the signature enrichment score calculation. See appendix (A3, Figure A.2) for constituent signature genes against CAR expression.



**Figure 5.5: CAR expression controls T cell dysfunction.** *Continued from the previous page.* **A)** Distribution of mean enrichment score for activation, dysfunction, and memory module signatures published by Singer et al [236] against increasing percentile rank, CAR expression bins. **B)** Regression analysis between gene signatures' averaged mean enrichment score and average DN-28z CAR per percentile rank, CAR expression bin. P value provided for all regression model fittings. Adjusted  $R^2$  provided for linear and logarithmic models. Approximated hill slope, lower limit, upper limit, and EC50 for 4-parameter log-logistic model fitting. P1, P2, ..., and P10 correspondingly represent Percentile Rank 1-10, Percentile Rank 11-20, ..., and Percentile Rank 91-100. **C)** Pearson's correlation analysis of mean enrichment score averaged per percentile rank, CAR expression bin against DN-28z CAR expression between donors A, B, C. Pearson's correlation coefficient and P value provided.

Interestingly the dysfunction module enrichment score distribution increasingly shifted against increasing CAR expression bins. This suggests that dysfunction, or exhaustion, increases with CAR expression. Taken further, this suggests that CAR expression could be tuned to control T cell exhaustion to a specific level. In contrast, the score distribution of both activation and memory modules begin initially at their highest levels but decreased with increasing CAR expression. This provides a further perspective on how CAR expression influences different T cell functions. Comparing activation and dysfunction modules together, the decrease in activation is consistent with observations demonstrating exhaustion impairing proliferative ability [152, 153, 166, 284-286].

Emboldened by these observations, I asked if there was a specific relationship or mathematical model that explains how exhaustion, activation, and memory vary with CAR expression. This insight would be beneficial by providing a means to identify whether certain CAR expression levels would predicate exhaustion and would be a powerful tool in addressing T cell exhaustion in CAR T therapies. To this end, I calculated the mean enrichment score for each CAR expression bin for each module. I then plotted them against CAR expression and conducted the previously described regression analysis (Figure 5.5b).

Regression analysis for the dysfunction module solidified my observations in the distribution of enrichment score shifting, with positive logarithmic and sinusoidal relationships best capturing the data points. Interestingly, the dysfunction module portrayed low enrichment scores at low CAR expression, from bins P1 to P3. However, after P3, an incline occurred before tapering off at CAR expression bin P8. This excitingly suggests that T cell dysfunction can be prevented by tuning CAR expression to lower levels. In contrast, the activation module portrayed robust scores in early CAR

expression bins P<sub>1</sub>-P<sub>3</sub>. However, it rapidly declined after P<sub>4</sub>, an inflection point, and continued with low scores. This suggests that after a certain CAR expression level, activation rapidly plateaus and T cells fail to proliferate. Taking both dysfunction and activation modules together, it appears that CAR expression bin P<sub>3</sub> is a key inflection point between the two behaviours. This suggests that until this point, T cells can undergo robust activation. However, past it, dysfunction takes over and impairs the key function. All models demonstrated weak fitting for the memory module; however, a negative sinusoidal relationship appeared to best capture points. Interestingly, the memory module demonstrated high enrichment scores at low CAR expression levels but after CAR expression bin P<sub>5</sub>, a rapid decrease occurs. This suggests an inflection point in memory function at higher CAR expression levels.

Seeking to confirm these findings, I conducted Pearson's correlation analysis between donors' dysfunction module mean enrichment scores. This analysis revealed a moderate-to-strong and significant correlation between all donors:

$$A \text{ vs } B: \rho = 0.87, P = 1.10 \times 10^{-6}$$

$$A \text{ vs } C: \rho = 0.82, P = 1.93 \times 10^{-5}$$

$$B \text{ vs } C: \rho = 0.68, P = 1.24 \times 10^{-3}$$

This confirmed that my observations between dysfunction and CAR expression translate amongst different donors and suggest the same translation for activation and memory.

Taken altogether, these findings provide compelling evidence that CAR expression levels strongly control exhaustion and weakly regulate activation and memory phenotypes. This indicates that it should be possible to reduce exhaustion by lowering CAR expression without compromising activation and memory capacity. While these findings require functional validation, they excitingly address a longstanding and critical problem within the CAR-T therapy field.

| Zheng 2021 Annotation         | General T cell subtype                          |
|-------------------------------|-------------------------------------------------|
| c01 $T_n$                     | Naive                                           |
| c02 $IL7^+ T_m$               | Memory                                          |
| c03 uncharacterised           | Clustered with memory cells in Zheng et al 2021 |
| c04 $ZNF683^+ CXCR6^- T_m$    | Memory                                          |
| c09 $KIR^+ TXK^+ NK$ -like    | NK-like                                         |
| c10 $ZNF683^+ CXCR6^+ T_{rm}$ | Resident Memory                                 |
| c12 Terminal $T_{ex}$         | Exhausted                                       |
| c13 $OXPHOS^- T_{ex}$         | Exhausted                                       |
| c14 $TCF7^+ T_{ex}$           | Exhausted                                       |
| c17 $NME^+ T$                 | See text                                        |

**Table 5.1:** Annotations of Zheng 2021 on tuned DN-28z CAR transcriptomic atlas

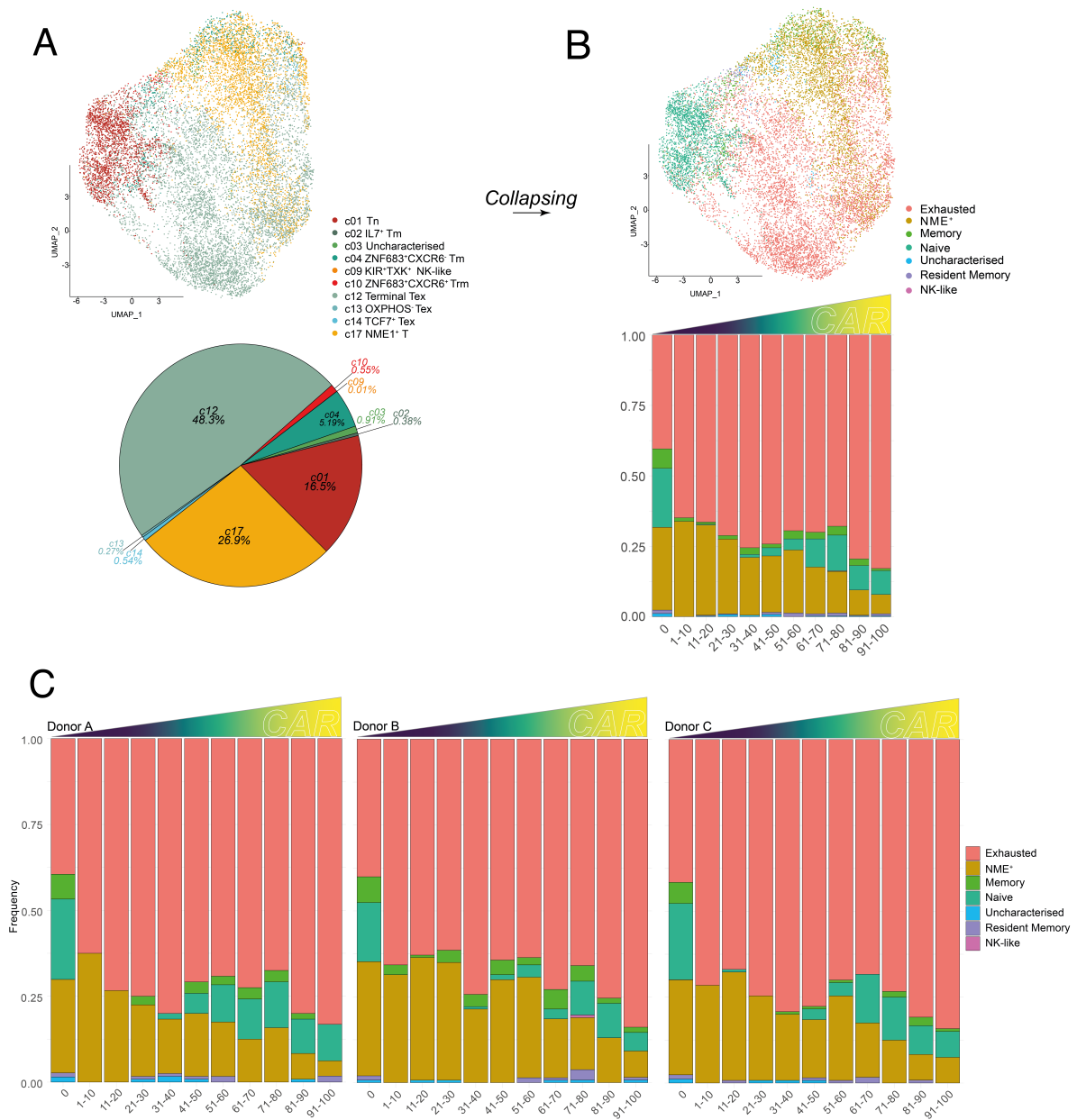
### 5.5 CAR EXPRESSION ELICITS RAPID INDUCTION OF EXHAUSTED T CELL SUBSETS

In the previous subchapter, I explored the impact CAR expression has on signatures related to T cell activation, dysfunction, and memory. T cells are a multifunctional cell type whose function is often associated and demarcated by differentiation state or lineage [158, 265, 291-293]. To this end, I asked what impact CAR expression has on the emergence of different T cell subsets, especially on exhausted and memory T cell lineages.

Recent efforts have documented the use of single-cell RNA-sequencing to construct atlases of T cells in a variety of contexts, including those infiltrating tumours and different organs or within the tumour microenvironment [238, 285, 294-299]. Zheng and colleagues [238] generated one such atlas from tumor-infiltrating T cells in 21 different cancers. They used transcriptional differences to finely distinguish the different T cell subsets of the more general exhaustion, memory, and naïve lineages. I leveraged their atlas, using Seurat’s anchor-based integration method [225], to understand the impact CAR expression has on T cell subset emergence, particularly on exhausted and memory subsets within  $CD8^+$  T cells.

Anchor-based integration led to 10 of the annotations being applied to CAR expression binned cells (Figure 5.6a), as seen in Table 5.1.

Terminal exhausted T cell (c12) led with the majority of annotations, labelling 48.3% of all cells,



**Figure 5.6: CAR expression rapidly gives rise to exhausted T cells.** **A)** Direct anchor transfer annotation [226] of Zheng and colleagues intratumoral T cell atlas [238] on all cells of DN-28z CAR-miSFTs scRNA-seq experiment. Top: UMAP visualization portraying localization of cell annotations Bottom: Pie chart depicting the composition of T cell subsets. **B)** Collapsing Zheng and colleagues anchor transferred annotations into more general T cell subtypes. Top: UMAP visualisation portraying localization of cell annotations. Bottom: composition of collapsed cell annotations by percentile rank, CAR expression bins. Bin '0' portrays CAR<sup>-</sup> cells, while 1-10, 11-20, ..., 91-100 portray CAR expression, percentile rank bins. **C)** Composition by collapsed cell annotations by percentile rank, CAR expression bins on an individual donor basis. Bin '0' portrays CAR<sup>-</sup> cells, while 1-10, 11-20, ..., 91-100 portray CAR expression, percentile rank bins.

CAR<sup>-</sup> and CAR<sup>+</sup>, while 26.9% of cells were annotated as NME<sup>+</sup> T cells (c17), and approximately 16.5% of cells were annotated as naïve cells (c01) (Figure 3.3.6a). NME1 expression was recently demonstrated by Szabo and colleagues [272] to be a marker of activation and tied to proliferation levels; thus, the NME<sup>+</sup> T cell annotation could represent activated T cells. Of memory subsets, 5.19% of cells were labeled as ZNF683<sup>+</sup>CXCR6<sup>-</sup> memory T cells (c04), while 0.38% were labeled as IL7R<sup>+</sup> memory T cells (c02). 1.04% were annotated as ZNF683<sup>+</sup>CXCR6<sup>+</sup> resident memory T cells (c10). Small percentages of cells were annotated by exhaustion subsets (c13, 0.27%, and c14, 0.54%).

To directly assess the impact of CAR expression on different T cell subset emergence, I collapsed the multiple subset variants into more general categories (e.g. exhausted, memory, resident-memory, naïve, and NME<sup>+</sup>) and calculated the frequency of each subset in each CAR expression bin (Figure 5.6b). I created expression bin '0' for cells undetected for *DN-28 CAR* expression; this bin was comprised of 10,096 cells.

Exhausted T cells was the major annotation for all CAR expression bins, exceeding 40% of all annotations. NME<sup>+</sup>, taken to be activated T cells, were the second most present annotation, appearing in all CAR expression bins. Naïve-like T cells, the third most present annotation, had a notable proportion in CAR<sup>-</sup> cells but only began appearing from expression bin 3 in CAR<sup>+</sup> cells. Memory T cell annotations were consistently present throughout all bins, albeit at low proportions. Similarly, resident memory T cell annotations appeared in all but three bins. Consistent with expectations, exhausted T cell annotations increased with increasing CAR expression, while NME<sup>+</sup> T cells decreased.

When comparing the composition of CAR<sup>-</sup> cells to the first CAR expression bin, there is a noticeable jump in the number of exhausted T cell annotations. More specifically, in CAR expression bin 0, 40% of annotations were exhausted T cells, while it was 65% in expression bin 1. Furthermore, there is a notable reduction in naïve and memory T cell annotations. This implies that CAR expression rapidly induces the emergence of exhausted T cell subsets while impeding memory subset maintenance.

Intriguingly, naïve T cell annotations peaked at CAR expression bin 7. It is likely these are not bona fide naïve T cells, but rather naïve-like cells, bearing key similarities in transcriptomic states. Further

investigation into these annotated cells would be beneficial in understanding what exactly T cell subset they are and what functionality they bear.

Reconducting the anchor-based integration of Zheng and colleagues' T cell atlas for each individual donors' cells confirmed our observations, with similar annotations and compositions being observed (Figure 5.6c).

Taken altogether, these results provide an interesting insight into how CAR expression impacts T cell subset emergence and composition. Consistent with expectations, increasing CAR expression gave rise to significant proportions of exhausted T cells at the expense of activated T cells. Even more, CAR expression elicited the rapid induction of exhausted T cells. These data provide preliminary evidence that an optimal composition of T cell subsets 'manufactured' via tuning CAR expression to specific levels, complementing my findings on preventing T cell exhaustion. However, this analysis would benefit from a more thorough assessment, incorporating clonotype tracking via TCR-sequencing [300, 301].

## 5.6 REVEALING GENES, REGULONS, AND PATHWAYS INFLUENCED BY CAR EXPRESSION

Thus far, my investigations have been guided with *a priori* knowledge, synergising previous findings by colleagues within the immunotherapy and immunogenomics communities. However, I asked what genes and signalling pathways were impacted with CAR expression that may have not been previously identified, as they may provide further mechanistic understanding in CAR-T based therapies.

To this end, I embarked on an unbiased discovery analysis. I leveraged mutual information, a quantification that measures how much information is shared between two variables, to identify genes whose expression levels vary in response to CAR expression [302-304]. I took my percentile rank-CAR expression bins, calculated the average expression of each gene, and calculated the normalized mutual information (NMI), a variant of mutual information akin to Pearson's correlation coefficient [243] to more easily identify genes whose expression varies strongly with expression of the *DN-28z CAR* transgene.



977 genes, 15% of genes within my single-cell expression matrix, were identified to have a normalized mutual information of 1, interpreted to have a 'perfect correlation' or 'complete informatic dependence', akin to Pearson's correlation [243], with *DN-28 CAR* expression (Figure 5.7a). Indeed, visualizing the average expression of all 977 genes confirmed tuned levels of expression in accordance with CAR expression (Figure 5.7b). Notable genes identified in this unbiased search include immune cell-related genes, such as interleukin receptors *IL7R*, *IL17RB*, and *IL21R*; major histocompatibility complex genes *HLA-A* and *HLA-DPB1*; and *ZFP36* and *ZFP36L2*, genes encoding RNA binding proteins demonstrated to have regulatory roles in CD8<sup>+</sup> effector function and differentiation [305-309]. These genes' expression patterns can be visualised in Appendix A.4, Figure A.3.

Intriguingly, visualising all mutual-information identified genes against increasing CAR levels revealed three modules of different expression patterns (Figure 5.7b):

**Top section:** A *positive* correlation module

**Middle section:** A negative correlation module whose expression *gradually* decreases

**Bottom section:** A negative correlation module whose expression *rapidly* decreases

I used PantherDB [410-12] to conduct gene ontology analysis to identify functional themes of each module. The first module failed to yield any gene ontology themes for all domains, even when expanding cutoff conditions. However, it contained transcripts encoding for transcription factors *HIVEP2* and *RUNX3*. *HIVEP2* has been shown to regulate a gene network and facilitate immunosuppressive roles within regulatory T cells [413]. *RUNX3* is an established transcription factor for T cells, active throughout T cell development, notably in CD8<sup>+</sup> T cell formation [414-5]. Recent evidence has shown it to have a role in T cell subset emergence and commitment. This includes effector T cells [416], resident memory T cells [417], and memory T cells [418].

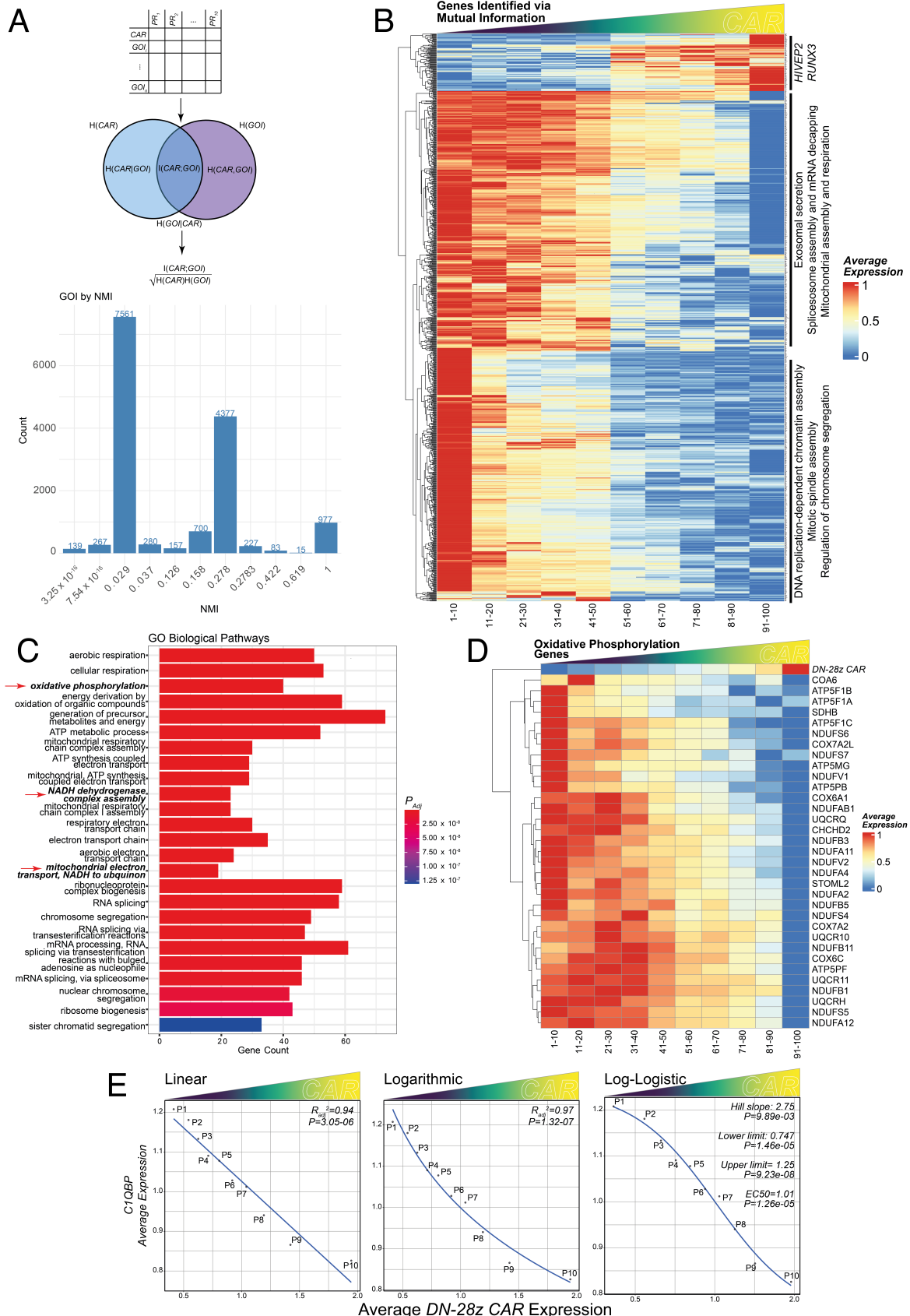


Figure 5.7: Mutual information identifies genes influenced by CAR expression. Continued on the following page.

**Figure 5.7: Mutual information identifies genes influenced by CAR expression.** *Continued from the previous page.* **A)** Top: schematic illustrating mutual-information protocol. In brief, DN-28z CAR and all other genes identified in single-cell RNA-sequencing were averaged using CAR expression bins. These average expression bins were then used for normalized mutual information calculation. Bottom: breakdown of normalized mutual information scores of all genes identified in single-cell RNA-sequencing experiment. **B)** Hierarchically clustered heatmap depicting average expression of all 977 genes identified to have a normalized mutual information of one, interpreted to have a complete dependence on or perfect correlation with CAR expression. Annotations denote gene ontology themes overrepresented for the gene module **C)** Gene ontology: biological pathways enrichment analysis of all 977 genes identified to have a normalized mutual information of 1. Y-axis depicts biological process, and x-axis conveys the number of genes of the process identified in normalized information. Colour fill portrays adjusted P values. Adjusted P values use Benjamini-Hochberg correction. Arrows spotlight oxidative phosphorylation and NADH pathways. **D)** Hierarchically clustered heatmap of genes of Gene Ontology: biological pathway of NADH dehydrogenase complex assembly. DN-28z CAR expression included for reference. **E)** Linear, logarithmic, and log-logistic regression analysis of C1QBP average expression across CAR expression percentile rank bins. Linear and logarithmic adjusted  $R^2$  and P values reflect model fitting. For log-logistic regression analysis, 4-parameter log-logistic function was used and portrayed on plots is hill slope, lower bound, upper bound, and EC50 and corresponding P value from model fitting. Both heatmap visualisations (**B,D**) portray average expression of each gene across CAR expression, percentile rank bins, represented by 1-10, 11-20, ..., 91-100. Average expression was scaled for visualization purposes such that gene's expression minima value was set to zero and the maxima set to 1. See Appendix A.4, Figure A.3 for heatmap containing 50 genes identified with 1 normalised mutual information.

The second module resulted in an enrichment of numerous biological processes including, exosomal secretion, 7-methylguanosine cap hypermethylation, U2-type prespliceosome assembly, mitochondrial electron transport, and proton motive force-driven mitochondrial ATP synthesis. Exosomes originating from CAR T cells have recently been demonstrated to have potent anti-tumour efficacy *in vivo* [413-14]. Furthermore, the U2-type prespliceosome is a component of the spliceosome, the protein complex responsible for removing introns from precursor mRNA [415-16]. 7-methylguanosine cap hypermethylation is necessary for the translation of mRNA [417]. Mitochondrial electron transport and ATP synthesis are involved in key metabolic processes for the generation of energy. This module demonstrated a gradual, negative expression pattern. Thus, this suggests CAR T cells are progressively unable to secrete exosomes, process RNA, and generate energy with increasing CAR expression.

Gene ontology analysis of the third module yielded biological processes DNA replication-dependent chromatin assembly, mitotic midzone assembly, and positive regulation of chromosome segregation. These processes are involved in the duplication and coordination of DNA in cell division [418-20]. Another process identified was protein-linked ubiquitination, the pathway that most commonly results in the degradation of proteins [421]. Mitochondrial translation was also identified; this process is

required for ATP production and the folding of mitochondrial cristae [422]. Furthermore, evidence has recently emerged, demonstrating mitochondrial translation to be essential in CD8<sup>+</sup> T cells for the translation of cytolytic proteins (e.g. perforin and granzyme B) and for sustained cytotoxicity [423-4]. This module portrayed a rapid decrease in expression against increasing CAR levels. This suggests that a CAR-T's ability to divide, degrade proteins and maintain protein levels, and elicit cytotoxic function to be swiftly impaired past low levels of CAR expression.

Taken altogether, these findings shed light on how unregulated CAR expression impacts several basic yet key cellular processes, either gradually or rapidly. They provide another side to T cell dysfunction, suggesting perhaps the source of dysfunctional T cell response. Indeed, cited evidence of this section [413-4, 423-4] corroborates this, connecting the dysregulation I observed here with the dysfunctional behaviour exhausted T cells have been associated with.

Importantly, when evaluating all modules' expression patterns, a 'goldilocks zone' of CAR expression can be suggested. In agreement with my previous findings in exhaustion and activation gene signatures (Figure 5.5), this zone is between CAR expression levels 1-30. In this zone, the genes of both negative correlation modules have appreciable expression levels before being severely impacted, and, thus, behaviour least impacted. While exciting, these results will require validation *in vitro* and *in vivo*, as discussed later in this chapter.

I next asked what processes are most impacted by CAR expression. To this end, I additionally conducted gene ontology analysis for all 977 genes identified through my mutual-information analysis. This resulted in numerous biological pathways related to metabolic processes, such as aerobic respiration, oxidative phosphorylation, and NADH dehydrogenase complex assembly, a protein of the electron transport chain [441] (Figure 5.7d). Visualizing average expression values of Oxidative Phosphorylation pathway-related genes confirms tuned levels of expression across CAR expression bins (Figure 5.7d). Intriguingly, these genes, encoding for multiple electron transport chain complex subunits [387-390], demonstrate a negative relationship with CAR expression. This suggests a dysregulation in the expression of these proteins necessary for metabolic function and processes against increasing CAR expression. Such findings merit further investigation and validation, using

gold-standard metabolic assays such as Agilent's Seahorse system [392].

I sought to explore these novel insights gained from my unbiased discovery analysis in more detail. To this end, I took a representative gene, *C1QBP*, and visualized its relationship with CAR expression. *C1QBP*, established to have a prominent role in mitochondrial oxidative phosphorylation, encodes Complement component 1Q subcomponent-binding protein. It has been recently shown by Zhai and colleagues to be essential for effector CD8<sup>+</sup> T cell differentiation [310-315]. Regression analysis revealed *C1QBP* has an inverse relationship with *DN-28z CAR* expression, suggesting that with increasing CAR expression, a decrease in mitochondrial oxidative phosphorylation would occur.

To complement these mutual-information-based results, I performed another unbiased search for genes that vary with CAR expression, this time using regression analysis. I used linear and logarithmic regression analysis given the ability to assess "goodness of fit" through the  $R^2$  metric. I once again took percentile rank-CAR expression bins, calculated the average expression of each gene, and conducted linear and logarithmic regression. I then assessed genes only of an  $R^2$  value greater than 0.9, as I wanted to only assess genes with a very strong correlation with CAR expression. Linear regression analysis revealed 221 genes with an  $R^2$  above 0.9, while logarithmic regression analysis revealed 365 genes with an  $R^2$  above 0.9 (Figure 5.8a,b). Of the 221 genes identified with linear regression, 132 (60% of 221) of them were identified by mutual information analysis. Of the 365 genes identified by logarithmic regression, 210 (57% of 365) were identified by mutual information analysis. This suggests that mutual information was able to capture an appreciable number of genes that have a relationship with CAR expression but there may be more, many of which may be well incorporated with lowering  $R^2$  cutoffs of linear and logarithmic regression, or by lowering the threshold for mutual information.

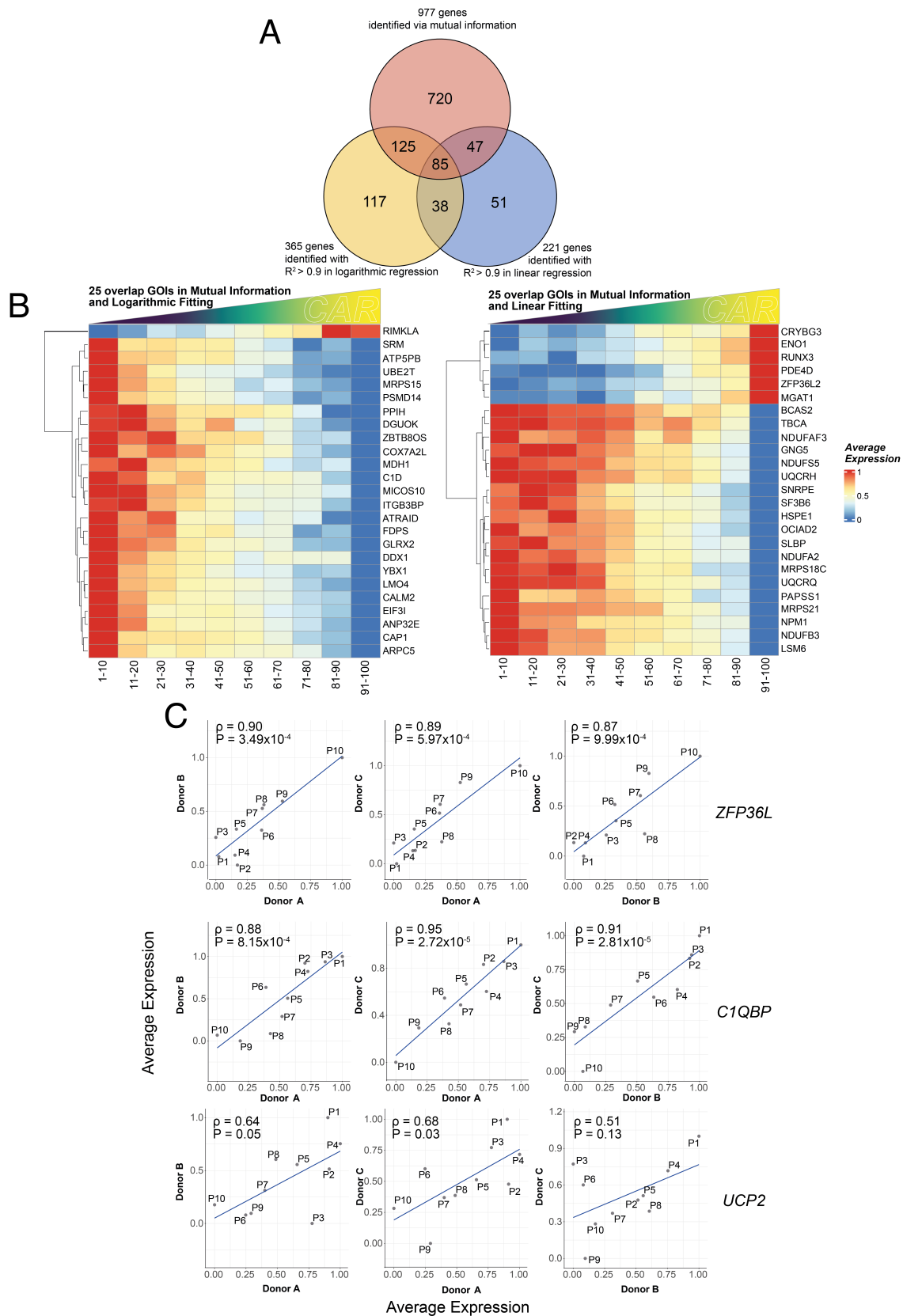


Figure 5.8: Regression analysis identifies additional genes influenced by CAR expression. *Continued on the following page.*

**Figure 5.8: Regression analysis identifies additional genes influenced by CAR expression.** *Continued from the previous page.* **A)** Venn diagram depicting overlap in genes identified by mutual information analysis and linear, and logarithmic regression analysis. Genes used in the analysis are those identified to have a normalized mutual information of 1, to have an  $R^2$  greater than 0.9, interpreted to have very strong correlation, in linear and logarithmic regression analysis. **B)** Hierarchically clustered heatmaps depicting average expression across CAR expression, percentile rank bins of a subset of genes identified to have an  $R^2$  greater than 0.9 in both logarithmic and linear regression fitting. 1-10, 11-20, ..., 91-100 portray CAR expression, percentile rank bins. Average expression was scaled for visualization purposes such that a gene's expression minima was set to 0 and maxima set to 1. **C)** Pearson's correlation analysis comparing average gene expression of *ZFP36L* (representing a linearly regressed gene), *C1QBP* (representing a logarithmically regressed gene), and *UCP2* (representing a mutual information identified gene) between single-cell RNA sequencing experiment component donors, A, B, and C. Pearson's correlation coefficient and P value provided. See Appendix A.4, Figure A.4 for heatmaps containing all genes identified with  $R^2$  above 0.9 in linear and logarithmic regression analysis.

Next, I asked whether the genes identified demonstrated similar relationships with CAR expression between different donors. To this end, I conducted Pearson's correlation analysis of the average expression of selected genes. I chose *ZFP36L*; encoding for zinc finger protein 36 L which has been shown to have roles in T cell activation, differentiation, and potency [442-4]; to represent a logarithmic gene identified in mutual information analysis. I used *C1QBP* to represent a linear gene identified in mutual information analysis. Finally, I selected *UCP2*, encoding for uncoupling protein 2, shown recently to have a role in regulating  $CD8^+$  metabolic reprogramming [445], as a gene identified purely through mutual information. This revealed a strong and significant correlation between all donors for *ZFP36L* and *C1QBP*, while *UCP2* demonstrated moderate correlation (Figure 5.8c). This suggests that the genes identified to have a relationship with CAR expression generally behave similarly between different donors.

As I observed genes encoding for transcription factors *HIVEP2* and *RUNX3* positively correlating with CAR expression, I next asked what transcription factors are additionally influenced by CAR expression. To this end, is used SCENIC to investigate what regulons (transcription factors and their downstream targets) are influenced by CAR expression. SCENIC analysis operates by synthesizing cis-regulatory sequence information with single-cell RNA sequencing to identify regulons with the highest activity scores within cells [239, 240, 242]. This revealed regulons whose activity scaled with CAR expression both positively and negatively (Figure 5.9a). Regulons identified related to T-cell effector function, differentiation, and regulation, notably including classical T-cell associated [316, 317] regulons (Figure 5.9a). These included nuclear factor of activated T cells family members, *NFATC2*

and *NFATC3*; nuclear factor- $\kappa$ B family members, *NFKB1* and *NFKB2*; RUNX family transcription factors *RUNX1*, *RUNX2*, and *RUNX3*; and *GATA3* and *MYB*. Interferon regulatory factor family members *IRF1*, *IRF2*, *IRF3*, *IRF4*, *IRF8*, and *IRF9* also portrayed regulon activity scaling with CAR expression. AP-1 members [318] *JUN*, *JUNB*, *JUND*, *ATF1-7*, *BATF*, *BATF3*, *FOS*, *FOSB*, and *FOSL1* also demonstrated activity that was influenced by CAR expression (Figure 5.9a). Lynn and colleagues [180] demonstrated *JUNB*, *FOSL1*, *BATF*, *BATF3*, *ATF3*, *ATF4*, and *IRF4* to be enriched in epigenetic exhaustion signatures, as well as have elevated protein expression, of CAR T cells. Regulons associated with dysregulation (e.g., *GATA3* [236], *BATF* [431-2]) increased their activity across CAR expression bins. These results further demonstrate the effects of tuned CAR expression on the T cell transcriptome.

I conducted regulon specificity score analysis, as demonstrated by Suo and colleagues [242], to identify regulons specific to each CAR expression bin (Figure 5.9b). This analysis revealed many previously identified regulons having activity in specific CAR expression bins. *MYB*, *BATF*, *BATF3*, *ATF4*, and numerous *E2F* transcription factors, demonstrated to have roles in proliferation [319-321], demonstrated specificity for lower CAR expression bins (Figure 5.9b). This observation complements my earlier observations in an activation gene signature (Figure 5.5).

In contrast, *FOSB*, *ATF5*, and *MAFF* demonstrated specificity for higher CAR expression bins (Figure 5.9b). Interestingly, *WRNIP1*, *FOSL1*, *RXRA*, and *NR2F6* demonstrated the highest specificity for the highest CAR-expression bin (Figure 5.9b). *WRNIP1* has been shown to have roles in DNA damage repair and regulatory T cells [322, 323]. *NR2F6* was demonstrated to correlate with *PD-1* and *CTLA-4* expression and act as an immune checkpoint by Klepsch and colleagues [324]; furthermore, the loss of *NR2F6* expression was demonstrated to enhance CD8<sup>+</sup> T cell memory formation by Jakic and colleagues [325]. Finally, *IRF9*, shown to have roles in preventing T cell exhaustion [289, 326], demonstrated a unique switch-like pattern, turning on in CAR expression bin 7 and turning off after (Figure 5.9b).



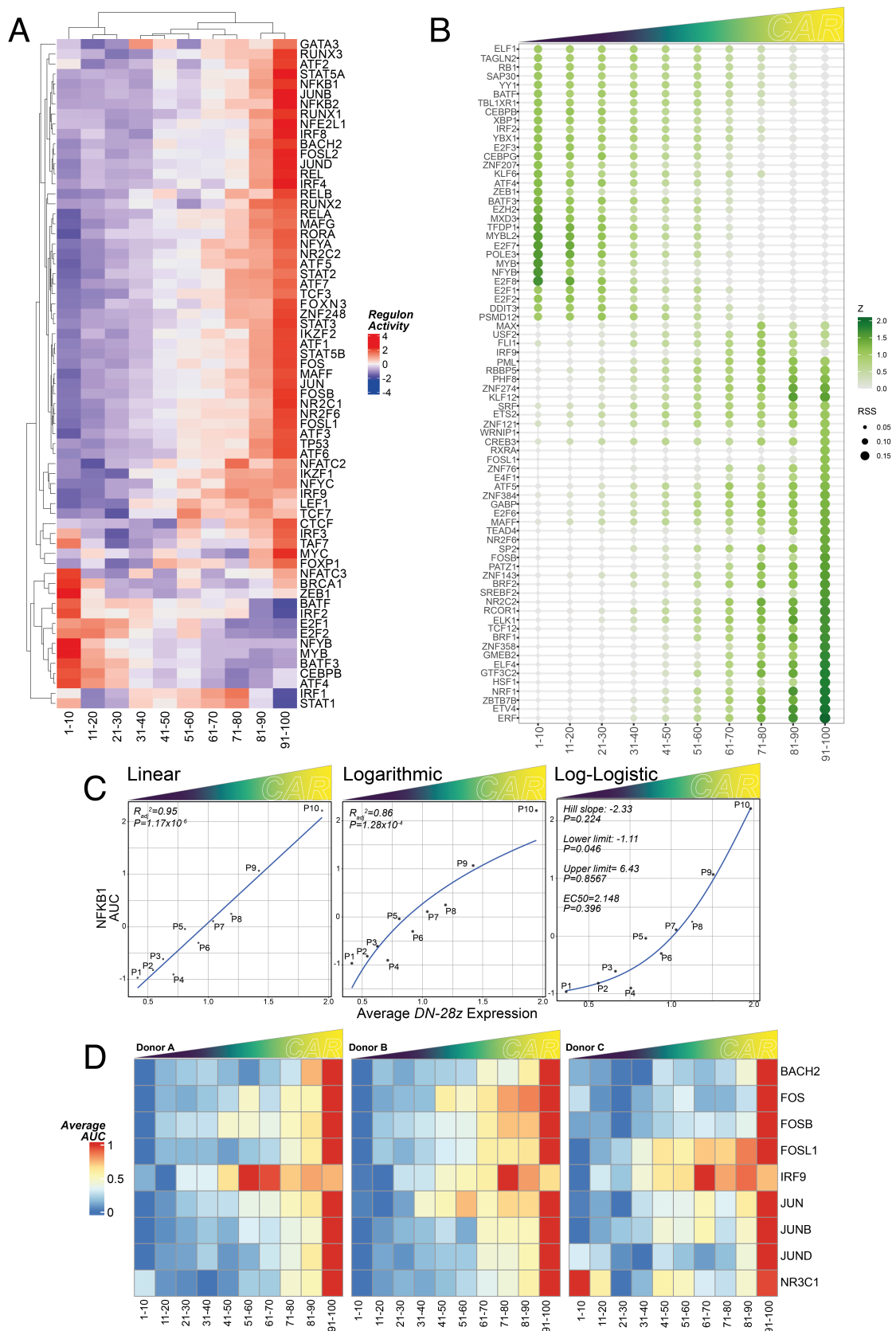


Figure 5.9: SCENIC analysis identifies CAR expression specific regulons. *Continued on the following page.*

**Figure 5.9: SCENIC analysis identifies CAR expression specific regulons.** *Continued from the previous page.* **A)** Hierarchically clustered heatmap depicting regulon activity score across CAR expression, percentile rank bins of a subset of regulons identified by SCENIC protocol to have highest regulon activity. Regulon activity score was scaled for visualization purposes such that the minimum value of a gene's expression was set to -2 and the maximum value set to 4. **B)** Dot plot depicting CAR expression, percentile rank bin specificity regulons. Dot size correlates to regulon specificity score, as described by Suo and colleagues [242]. Colour fill depicts z-score. **C)** Linear, logarithmic, and log-logistic regression analysis of average regulon activity score of *NFKB1* across CAR expression, percentile rank bins. Linear and logarithmic adjusted  $R^2$  and P values reflects model fitting. For log-logistic regression analysis, 4-parameter log-logistic function was used and portrayed on plots is hill slope, lower bound, upper bound, and EC50 and corresponding P value from model fitting. **D)** Average regulon activity score of regulons *BACH2*, *FOS*, *FOSB*, *FOSL1*, *IRF9*, *JUN*, *JUNB*, *JUND*, and *NR3C1* across CAR expression, percentile rank bins. 1-10, 11-20, ..., 91-100 portray CAR expression, percentile rank bins. P1, P2, ..., P10 correspondingly represent Percentile Rank 1-10, Percentile Rank 11-20, ..., and Percentile Rank 91-100. See Appendix Appendix A.4, Figure A.5 for heatmap containing all Regulons identified in SCENIC analysis.

I once again sought to validate my findings via regression analysis, assessing the relationship regulon *NFKB1* has with CAR expression (Figure 5.9c). This analysis revealed that a linear mathematical model best described *NFKB1*'s relationship with *DN-28z* CAR expression. This suggests CAR expression directly influences *NFKB1*, which is unsurprising given that the *NFKB* transcription factor family has roles in T cell activation, as previously described [249, 254]. Intriguingly, this runs in contrast with my previous observations in Jurkat T cell models; however, this difference is potentially explained by model observations being within a genomically integrated reporter for NFkB, thus impacting activation rates.

I sought to further confirm regulon activity by assessing the activity of *BACH2*, *FOS*, *FOSB*, *FOSL1*, *IRF9*, *JUN*, *JUND*, and *NR3C1* amongst the different T cell donors (Figure 5.9d). This demonstrated similar expression patterns, confirming that these regulons' activity varies over CAR expression in a similar fashion across donors.

Taken together, these results demonstrate further compelling evidence of CAR expression's impact on the T cell transcriptome. When considered with the genes from my mutual-information analysis (Figure 5.7), it is possible that the transcription factors I identified are the potential drivers of the dys-regulated expression patterns observed. Indeed, *RUNX3* was identified in both analyses (Figure 5.7b and 5.8a). In both analyses, it demonstrated to positively correlate with CAR expression. Thus, it can be suggested to be a potential driver in T cell dysfunction. This analysis can be extended by assessing all identified regulons and their targets to create a gene regulatory network (GRN). This GRN could

then be used to explain the previously observed expression profiles and potential dysregulated behaviours (Figure 5.7b), thus constructing a gene dysregulatory network. However, while these observations and ideas are exciting, regulon analysis is based on transcriptomic, co-expression analysis and thus, such observations would need to be confirmed with gold-standard techniques, such as single-cell ATAC sequencing [333, 334] and ChIP [364].

I next sought to identify which biological pathways were influenced by CAR expression. To this end, I reconducted my mutual information analysis, first calculating the enrichment score of all 3,825 *DN-28z CAR* expressing cells for BioCarta pathways. I calculated the average expression per CAR expression bin and calculated the normalized mutual information (NMI) between each BioCarta pathway enrichment score and *DN-28z CAR* expression. I identified 86 BioCarta pathways that are associated with *DN-28z CAR* expression (Figure 5.10a).

Pathways associated with T cell activity and behaviour were among those that strongly associated with CAR expression (Figure 5.10a, bottom). This notably includes T cell activation-related signalling pathways: protein kinase C (PKC) [327],  $\text{Ca}^{2+}$ -Calmodulin protein-kinase activation (PKA) [328], p38 mitogen-activated protein kinases (p38-MAPK) [433], nuclear factor for activated T cells (NFAT) [434-5], Calcineurin [436], and T cell receptor (TCR). Additionally, pathways associated with immune responses, including the innate immunity classical complement system [437-8], the antigen presentation system major histocompatibility complex (MHC) [439-40], and T cell cytotoxicity. Indeed, visualizing the average expression of the T-cell cytotoxicity pathway's component genes demonstrates a clear variation of expression levels against CAR expression bins (Figure 5.10b). T cell cytotoxicity demonstrated a reduced enrichment score against increasing CAR expression bins, corroborating previous results demonstrating dysfunction impairing T cell effector function [99, 288, 329].

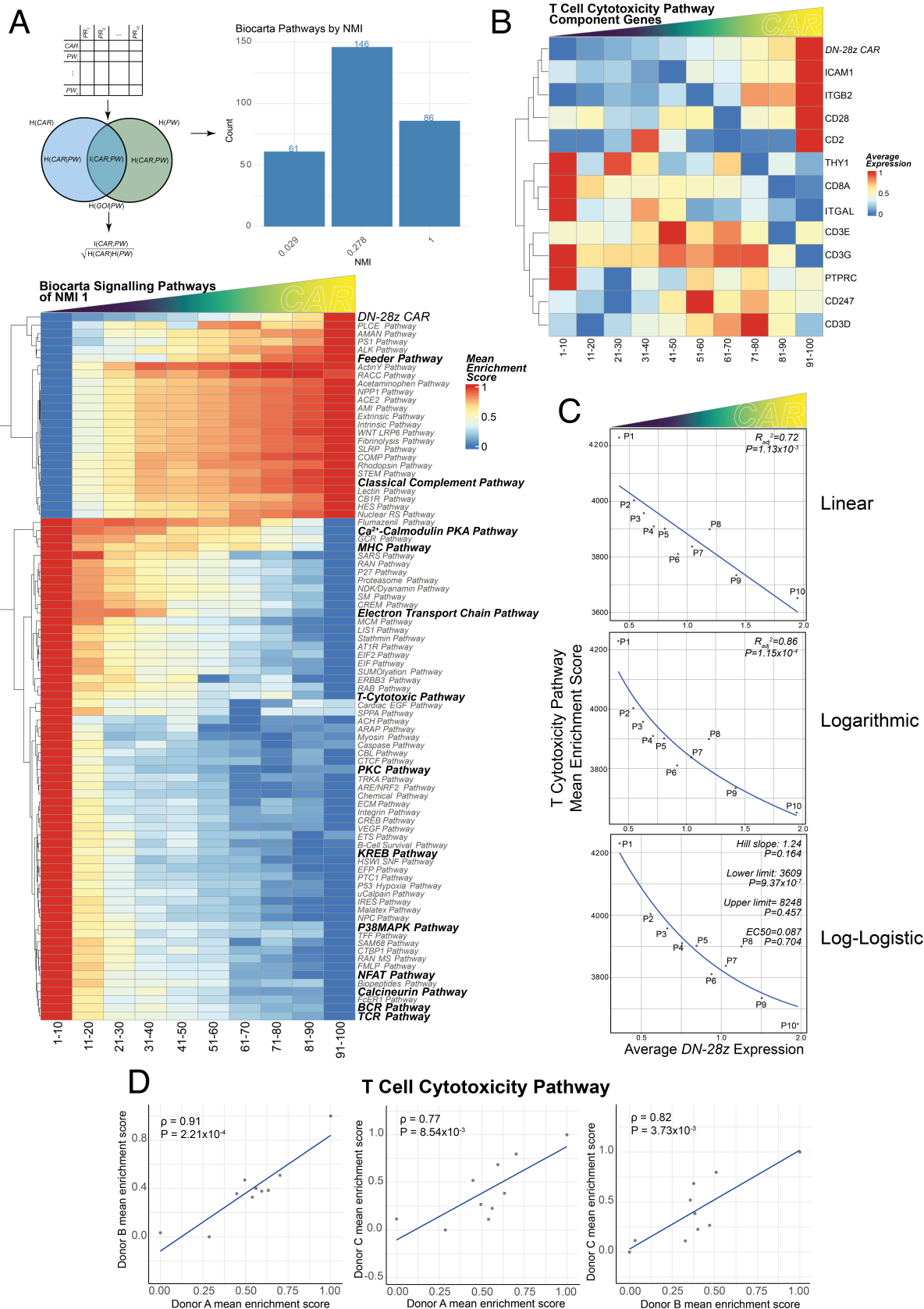


Figure 5.10: Signaling pathways impacted by tuned CAR expression. Continued on the following page.

**Figure 5.10: Signaling pathways impacted by tuned CAR expression.** *Continued from the previous page.* **A)** Top: Left, schematic representing normalised information pathways. Right, Biocarta pathways by normalized mutual information values. Normalised mutual information of 1 is interpreted to be a perfect correlation. Bottom: Hierarchically clustered heatmap depicting average enrichment score across CAR expression, percentile rank bins of Biocarta pathways identified with normalized mutual information of 1. *DN-28z* CAR expression included for reference. **B)** Hierarchically clustered heatmap depicting average expression across CAR expression, percentile rank bins of genes composing Biocarta signalling T cell cytotoxicity pathway. *DN-28z* CAR expression included for reference. **C)** Linear, logarithmic, and log-logistic regression analysis of Biocarta T cell Cytotoxicity average enrichment score across CAR expression, percentile rank bins. Linear and logarithmic adjusted  $R^2$  and P values reflect model fitting. For log-logistic regression analysis, 4-parameter log-logistic function was used and portrayed on plots is hill slope, lower bound, upper bound, and EC50 and corresponding P value from model fitting. P1, P2, ..., P10 correspondingly represent Percentile Rank 1-10, Percentile Rank 11-20, ..., and Percentile Rank 91-100. A-C) 1-10, 11-20, ..., 91-100 portray CAR expression, percentile rank bins. Average enrichment score was scaled for visualization purposes such that the pathway enrichment score's minima was set to zero and the maxima value set to 1. **D)** Pearson's correlation analysis comparing Biocarta T cell Cytotoxicity pathway average enrichment score between single-cell RNA sequencing experiment component donors, A, B, and C. Pearson's correlation coefficient and P value provided.

Surprisingly, the TCR pathway decreased against increasing CAR expression, contrary to expectations. This is because as CAR expression increases, therefore the number of CARs increases. Thus, this increases the opportunities for a CAR to bind and signal, leading to the increased activity of genes involved in the TCR pathway. However, this decrease is in agreement with our previous observations in the dysfunction of T cell activation (Figure 5.5) and reiterates the need for further investigation in functional tests.

Additional pathways identified included metabolic signalling pathways, complementing my earlier findings (Figures 5.7 and 5.8). This includes electron transport chain (ETC) pathway, Krebs cycle pathway, and feeder pathways for glycolysis. Intriguingly, feeder pathways for glycolysis clustered closely with and demonstrated a positive expression pattern with *DN-28z* CAR expression. This suggests that with increasing CAR expression, T cells begin to rely on glycolysis more as a source for energy. In contrast, both ETC and KREB cycle pathways demonstrated a negative relationship with CAR expression. These findings add confidence to my previous observations in the enriched gene ontology themes associated with my mutual-information genes (e.g., mitochondrial translation and NADH dehydrogenase complex assembly, Figure 5.7), as well as genes themselves (e.g., *CIQBP* and *UCP2*, Figure 5.7 and 5.8). Furthermore, it has been shown that CARs rewire metabolic pathways based on which costimulatory domain used in CAR structure[67]; however, this adds to such knowledge by demonstrating how another design parameter impacts CAR T cell metabolism. Once again,

these observations, while exciting, will require robust functional confirmation, as previously mentioned (e.g., Seahorse). This analysis demonstrates how CAR expression influences multiple signalling pathways.

I next explored the T cell cytotoxicity pathways relationship with CAR expression in more detail. Regression analysis revealed an inverse relationship (Figure 5.10c), suggesting a reduction in effector capacity against increasing CAR expression, consistent with my previous observations.

Pearson's correlation analysis of the mean enrichment score of cytotoxicity pathway revealed strong and robust correlation between all donors, confirming these observations translating between donors (Figure 5.10d):

$$A \text{ vs } B: \rho = 0.91, P = 2.21 \times 10^{-4}$$

$$A \text{ vs } C: \rho = 0.77, P = 8.54 \times 10^{-3}$$

$$B \text{ vs } C: \rho = 0.82, P = 3.73 \times 10^{-3}$$

Taken together, these results demonstrate the clear impact CAR expression has on multiple signalling pathways related to T cell function and activity. However, similar to regulon analysis, these results require further validation utilising phosphoproteomic experiments to finely assess phosphorylation of targets along signalling paths.

## 5.7 DISCUSSION

Once again, the main questions guiding my DPhil are as follows:

1. Is it possible to tune CAR expression to precise levels of expression utilizing the miSFITs tuning system?
2. If so, how do CAR expression levels impact T cell activation and the transcriptome?

Addressing question one, I demonstrated tuned CAR expression on a wider scale, using the entire DN-28z CAR-miSFITs library, advancing my observation in T cell models (Fig 5.1 and 2). Leveraging this atlas of tuned CAR expression, I undertook a guided analysis, synthesizing the results of others

to provide crucial insights on T cell transcriptional identity and to probe challenges dominating the CAR-T field. Notably, I demonstrated that tuned CAR expression impacts the expression of several T cell marker genes related to activation, memory capacity, and dysfunction (Figure 5.3). Extending these transcriptomic insights further, I demonstrated inflection points within CAR expression where T cell activation, dysfunction, and memory capacity change (Figure 5.4). I synthesize these data, suggesting how CAR expression could be optimized to maximise activation, effector, and memory phenotypes while reducing T cell exhaustion. Throughout this analysis, I leveraged regression fitting to guide my interpretation of these data and to suggest relationships these genes and signatures have with tuned CAR expression (Figure 5.5 and 5.6). These data provide answers to question two of my DPhil. Further, I investigated the impact of tuned CAR expression against T cell subset emergence, demonstrating rapid induction of exhausted T cells at the decline of activated and memory T cells (Figure 5.6).

I then conducted an unbiased analysis in which I identified genes, both novel and known to be related to T cell function, influenced by CAR expression. This analysis notably identified numerous genes related to several essential cellular functions related to RNA processing, cell division, and T cell metabolism (Figure 5.7 and 5.8). I complemented this analysis, using regression analysis to identify additional genes associated with CAR expression. I continued this analysis by identifying regulons specifically active in specific CAR expression bins (Figure 5.9) and characterizing the relationship regulons previously associated with memory and dysfunction have with CAR expression. I concluded by identifying numerous signaling pathways associated with CAR expression, including those related to T cell activation and metabolism (Figure 5.10). When evaluating cell markers and gene signatures related to T cell activation, memory, and dysfunction, I visualised responses against tuned levels of CAR expression (Figure 5.3, 5.4, 5.5). This analysis could be extended by including the variable of time. Doing so would create a more representative picture of CAR-Ts when used *in vivo*.

Even more, this analysis would provide insight as to whether what was being tuned: the genes and behaviour itself or the timing of the behaviour (e.g., activation vs the time to activation). More specifically, I demonstrated in Figures 5.4 and 5.5 the relationships of cell markers with CAR expression.

However, the time analysis would add a third, orthogonal dimension. This would allow a more representative picture of CAR-T cell behaviour as it would be used in a therapeutic setting. For example, at lower CAR expression levels, I observed the exhaustion gene signature at its lowest level. However, these lower points may rise with increasing time. One way this analysis could be conducted is through the use of pseudotime trajectory inference methods with my CAR expression atlas using established methods, such as Monocle3 [407], Slingshot [408], or Wishbone [409].

However, these methods may provide a limited conclusion, given they would be inferences based on transcriptomic data, generated from T cells over a very short time period (i.e., a 24h co-culture). Thus, a more appropriate way to evaluate the impact of time would be to conduct long-term (e.g., 1-2 week) co-cultures, creating RNAseq libraries or measuring cell markers at various time points (e.g., 24h, 48h, 5 days, 10 days, etc.) as has been demonstrated previously in organoids [446].

When investigating the impact of CAR expression level on T cell subset emergence, I used the cells of my CAR expression atlas with 0 *DN-28z* CAR expression as a bin to represent CAR<sup>-</sup> cells. However, it is unlikely that all the cells of this created bin truly have 0 *DN-28z* CAR expression. This could be explained by library preparation (e.g. failure to complete all RNA transcripts within a 10X droplet) and sequencing limitations, such as sequencing drop-out. Thus, the exact subset composition remains to be confirmed and the associated analysis requires validation, for example using the gold standard method of flow cytometric immunophenotyping via subtype markers [447-8].

As noted earlier, multiple T cell atlases have been published [238, 285, 294-299], each demonstrating a variety of annotations. Thus, these findings would benefit by being compared against the annotations generated using the previously described anchor-transfer analysis with these other atlases.

It should be noted that these suggested, aforementioned anchor-transfer-based analyses are guided by *a priori* knowledge, using a selected dataset. Thus, this analysis would benefit by utilising packages that perform unbiased cell type recognition, including but not limited to CellTypist [384], SingleR [385], and SingleCellNet [386].

In my unbiased analysis, I used mutual information and regression fitting to identify genes that have relationships with CAR expression. Comparing these analyses, I noted an appreciable number



of genes uniquely identified by regression analysis and undetected by mutual information: 42% of 365 for logarithmic regression and 40% of 221 for linear regression. One potential explanation for this is the cutoffs established for mutual information analysis. I set a cut-off of a normalized mutual information value of 1, representing a ‘perfect’ correlation, and an  $R^2$  of greater than 0.9, representing a very strong correlation, to balance false positives and false negatives in identification. Further analysis would benefit from optimizing both normalised mutual information and  $R^2$  cutoffs.

Both mutual information and regression analysis were able to identify an appreciable number of genes influenced by CAR expression: 977 using mutual information 365 using logarithmic regression, and 221 using linear regression. Mutual information clearly identified the largest number of genes but, taking into consideration the above discussion, did not identify all genes identified by linear and logarithmic regression. However, mutual information can identify relationships outside specified regression analysis. Thus, mutual information analysis serves as a good ‘first-pass’ filter to identify potential genes of interest but greatly benefits from regression analysis to confirm and build upon the genes identified. Indeed, selecting specific regression analysis, motivated by the observations that arise from mutual-information analysis, as seen here, is an efficient strategy to quickly identify genes, potentially clusters of genes, impacted by CAR expression.

I used single-cell RNA sequencing to understand T cell response tuned CAR expression. I chose single cell RNA sequencing due to its ability to resolve heterogeneity within a population and to directly relate CAR expression to transcriptomic response. However, these findings would greatly benefit from further validation experiments, utilizing single-cell TCR sequencing [330-332] (to validate my observations in T cell subset emergence), single-cell ATAC sequencing [333, 334] (to validate my observations in regulon analysis), phosphorylation/phosphoproteomic assays [72] (to validate my observations in pathway analysis), and protein-based large-scale mass cytometry [335-337] and CITE-seq [338].

Functional data, most importantly *in vivo* mouse models and killing assays, are necessary experiments to understand how these transcriptional properties might impact the key T cell functions they underpin. In analyzing gene signatures, I discovered that lowering CAR expression with miSFITs

could mitigate exhaustion while maintaining essential functions such as T cell activation and memory function. Functional data to assess whether this translates to cell function and behavior is the essential next step. These findings would greatly address an unmet need within the CAR-T community.

In essence, in this subchapter I provide key evidence confirming CAR expression's influence over the T cell transcriptome. I demonstrated compelling data to suggest that tuning CAR expression can be used to combat T cell dysfunction without compromising T cell activation. I showed how CAR expression rapidly gives rise to exhausted T cell populations. Finally, I identified genes, regulons, and pathways related to key, fundamental cellular processes, such as T cell metabolism and cell division, influenced by CAR expression.

# 6

## Conclusion

The over-arching goal of my doctoral work was to dissect the impact of tuning chimeric antigen receptor expression in human T cells. More specifically, this work was guided by two main questions. The first: is it possible to tune chimeric antigen receptors in human primary T cells? And the second: how do tuned CAR expression levels impact the T cell activation and transcriptome?

In this thesis, I document my efforts to tackle these guiding questions. I adapted the miSFITs tuning system to tune the expression of NY-ESO-1-targeting DN-28z CAR within T cell models and human primary T cells. I then demonstrated how tuned CAR expression correspondingly tunes multiple genes, regulons, and pathways of the T cell transcriptome, thereby answering the guiding questions of the project.

I then leveraged these findings to visualise potential mathematical models that would explain CAR expression's influence on genes, regulons, and pathways. Finally, I harnessed these visualisations to suggest how tuned CAR expression may be used to tackle outstanding issues hamstringing CAR-T therapies.

In this chapter, I consider the limitations of my investigations that still remain to be addressed and describe key work that needs to be done before these findings could be translated into current CAR-T therapies. This includes the need for functional testing and how I use miSFITs sensitive towards miR-17, which has important roles in T cell functions [245, 340-345]. I will also highlight how my findings can be used to address outstanding research challenges.

## 6.1 CONSIDERATIONS AND CURRENT LIMITATIONS OF DN-28z CAR-miSFITs

In this work, I adapted a miSFITs library responsive to microRNA-17 (miR-17) to tune the expression of a CAR variant. I chose this library because miR-17 is expressed in T cells and the many subsets [244, 339, 341-5, 393-7] and because miR-17 miSFITs have previously undergone extensive characterisation and validation [219]. However, miR-17 has been demonstrated to play functional roles in T cells [340-5, 393-7], including involvement in proliferation [245], development [342], and differentiation [343]. More recently, miR-17 has also been implicated in the suppressive behaviour of the regulatory

T cell subset [345]. These key T cell behaviours may be impacted by introducing miR-17 response elements. Moreover, other miRNAs of the miR-17 family or miR-17-92 cluster, that present a certain degree of similarity to the canonical miR-17 [346, 347] and have putative roles in T cells as well [341, 343, 344] could also be impacted by the presence of MREs found in CAR-T constructs [341, 343, 344]. However, Michaels and colleagues [219] have clearly demonstrated that miR-17 MREs do not act as sponges, and their presence is unlikely to impact/suppress normal functions of miR-17-related species. However, Michaels and colleagues demonstrated a weak correlation in tuning between miR-17 and miR-21 miSFITs. As miR-21 is not relative of miR-17, this finding highlights the importance of assessing the effects of exogenous MREs on other miRNAs expressed in T cells at the time of CAR T delivery and action.

Regardless, adapting a miSFITs library responsive to another miRNA not associated with any key T cell behaviours may be required for further investigations in T cells. As previously described [219], it is possible to generate and validate a new miSFIT library against another miRNA in under two weeks. Thus, addressing this limitation is straightforward and possible within a reasonable time frame.

In preliminary tests, I used the miSFITs library to tune the NY-ESO-1 targeting DN-28z CAR. I selected this CAR due to NY-ESO-1's specific expression in cancer cells and male germ cells. Furthermore, this specific CAR variant uniquely bound HLA presented NY-ESO-1 antigen, with specificity akin to the endogenous T cell receptor [30]. In my experiments, I demonstrated the robust tuning of this CAR to precise, step-wise expression levels. However, it is uncertain whether this tuning translates to other CAR variants and further testing will be needed when designing a new CAR strategy targeting other antigens and CARs with different modular components.

In a similar vein, I leveraged tuned CAR expression to investigate the impact on the T cell transcriptome. CARs are modular proteins, and evidence has shown differences in behaviour and signalling pathway usage when changing co-stimulatory molecules of the intracellular domain [54, 349, 350]. The observations in tuned DN-28z CAR expression may not translate to CARs of different architectures. Therefore, it is likely the transcriptomic assessment I conducted would need to be repeated for each specific CAR.

Within my tuned CAR expression transcriptomic atlas, I observed intriguing insights related to genes, regulons, and pathways. However, these observations are based only on transcriptomic data. They do not provide evidence on T cell behaviour and functional response. As previously discussed in Chapter 3.3, confirmation studies featuring proteomic and functional experiments would be needed to validate such findings. Thus, they could potentially translate to current CAR-T therapies and serve as a basis for further investigations.

In summary, the data presented in this thesis provide notable insights into T cells' response to tuned levels of CAR expression. In chapter 3, I demonstrated how tuning CAR expression can tune the CAR's sensitivity to antigen, thereby controlling the magnitude of activation and the number of cells activated (Figure 3.6). In chapter 4, I showed how tuning CAR expression can control CD69, an early activation marker, expression levels (Figures 4.2 and 4.3). In chapter 5, I showed that tuning CAR expression provides a potential way to abate T dysfunction without compromising optimal T cell activation (Figure 5.5). Moreover, I showed how CAR expression, even at its lowest level, rapidly increases the proportion of exhausted T cells within a population (Figure 5.6). I also showed that CAR expression directly influences numerous genes, pathways, and regulons (Figures 5.7, 5.8, 5.9. and 5.10), and have specifically demonstrated that CAR expression directly tunes oxidative phosphorylation-related genes (Figure 5.7). However, further work is required to investigate whether these findings apply to all CARs. Hence, considerable validation work is still required before being in a position to translate these insights into current CAR-T therapies.

## 6.2 NOVELTY AND ADVANTAGES

Recent studies have investigated the potential impact of CAR expression levels on T cell behaviour. In one strategy, Walker and colleagues [351] titrated retroviral supernatant to achieve three different levels of expression of two CAR variants: CD19 and ALK targeting CARs in PBMCs. CD19 is a gold-standard B-cell marker that has been targeted in successful CAR-T therapies, while ALK, anaplastic lymphoma kinase, is a potential target overexpressed in pediatric solid tumors. For ALK CARs, the

| ALK CAR expression [gMFI] | CD19 CAR expression [gMFI] |
|---------------------------|----------------------------|
| 1213                      | 3287                       |
| 1685                      | 10715                      |
| 1950                      | 18397                      |

**Table 6.1:** Walker and colleagues tuning of CAR expression

authors were able to moderately tune CAR expression: 1213, 1685, and 1950 gMFI, resulting in a fold change of 1.2x. For CD19 CARs, they more dramatically tuned CAR expression: 3287, 10715, 18397 gMFI, resulting in a fold change of 5.6x. These are shown in Table 6.1.

While it is likely Walker and colleagues can attain further expression levels by increasing the amount of retrovirus applied, Eyquem and colleagues [99] demonstrated that CAR expression delivered by a single retroviral particle predisposed T cells towards exhaustion. Thus, adding more virions to deliver more CAR transgene would accelerate this process. In contrast, tuning down the baseline CAR expression would be more appropriate. My observations in gene signatures (Figure 5.4) demonstrate this, as dysfunction increased with CAR expression.

Additionally, Walker and colleagues' tuning strategy demonstrated different degrees of tuning. One potential explanation of this difference could be related to the type of retrovirus generated and how amenable each CAR was to being transduced. Regardless, these findings underscore the point raised earlier on the need to validate the portability of tuning systems between different CARs.

However, Walker and colleagues were able to gain notable insights into functional CAR T response using their tuned CAR expression models. For both ALK and CD19 CARs, they stimulated their three lines of expression, referred to as low, medium, and high in terms of T cell activation by co-culturing them with increasing concentrations of target antigen. Their cells contained an NFAT-GFP reporter, akin to my NF- $\kappa$ B reporter system, that produced GFP, green fluorescence protein, as a read-out for the activity of NFAT, Nuclear factor of activated T-cells, a key transcription factor involved in T cell activation. The authors measured GFP produced in response to antigen exposure. For both CARs, they observed step-wise activation levels in response to increasing antigen concentrations in both medium and high CAR-expressing cells, akin to my observations in Jurkat T cell models. In-

Interestingly, low-CAR expressing cells rapidly reached a plateau of NFAT-GFP response. When measuring cytokine production in response to antigen stimulation, low CAR-expressing cells produced drastically reduced amounts of cytokines, near-zero, in comparison to cells with medium and high expression CARs. These results are in line with my observations that high CAR expression miSFITs (Scramble, V<sub>12</sub>, V<sub>14</sub>) also displayed step-wise levels of NF- $\kappa$ B-EGFP expression at high NY-ESO-1 peptide concentrations (Figure 3.6). Furthermore, low CAR expression miSFITs (4x Perfect, 2x Perfect, 1x Perfect) demonstrated rapid plateauing in NF- $\kappa$ B-EGFP expression at high peptide concentrations.

Furthermore, Walker and colleagues' tuning of ALK and CD19 CARs was notably different, both in step-wise levels and range. This provides evidence that tuning CAR expression may be CAR dependent, as discussed in 4.1.

Juillerat and colleagues [355] demonstrated another method of tuning CAR expression by coupling a CD22 targeting CAR with a degradation moiety (degron) and employing a protease strategy. CD22 is an adhesion molecule restricted to B cell lineages that has demonstrated remarkable promise as an alternative to CD19-based therapies [356]. For their protease strategy, Juillerat and colleagues used the Hepatitis C virus (HCV) NS<sub>3</sub> protease and its inhibitor Asunaprevir (ASN) to control CAR protein levels. The HCV NS<sub>3</sub> protease is a protease with roles in the maturation of the viral polyprotein [357], while ASN is a recently developed competitive inhibitor demonstrated to robustly block HCV NS<sub>3</sub> activity [358-360]. They fused the HCV NS<sub>3</sub> protease, HCV NS<sub>3</sub> target site, and a degron into the C-terminal end of the CAR. In their strategy, the presence of ASN inhibited the cleavage of the degron from the CAR by the HCV NS<sub>3</sub> protease, leading to the degradation of the CAR. Juillerat and colleagues used this strategy to tune CAR expression to five levels using a titration of ASN, as shown in Table 6.2.

Their strategy used required the constant application of an external drug while a patient would receive treatment with CAR-T therapy. The miSFITs tuning system would offer a unique advantage, given that gene tuning is genetically encoded by each MRE. This results in stable tuned, expression without the need for external intervention. Moreover, the NS<sub>3</sub> protease is virally-derived, and thus



| ASN Concentration [nM] | MFI, as approximated from plot |
|------------------------|--------------------------------|
| 0                      | 2400                           |
| 100                    | 1500                           |
| 200                    | 1400                           |
| 200                    | 1400                           |
| 500                    | 1100                           |
| 1000                   | 1150                           |

**Table 6.2:** Juillerat and colleagues tuning of CAR expression

could potentially evoke an immune response, severely limiting any CAR-Ts infused. Finally, Juillerat and colleagues did not observe any significant effects of ASN on T cell proliferation, viability, or ability to secrete cytokines, and thus the impact of their approach on other human cells remains uncertain.

However, Juillerat and colleagues used this system to assess tuned CAR expression's impact on cytolytic function. They co-cultured CAR-Ts of 6 different expression levels with target cells expressing both the CAR's antigen and luciferase. They monitored the percentage of target cells surviving co-culture by measuring luciferase expression. They found that tuned levels of % killing corresponded to CAR expression, where the highest expression resulted in the highest % of target cells killed and vice versa. Furthermore, they also found that tuning CAR expression also correspondingly tuned correlated secretion of cytolytic factors. I made observations in my studies, for example, *PRFI*, encoding perforin which forms pores in cell membranes [367] demonstrated an expression pattern that closely correlated to that of the *DN-28z CAR* (Figure 5.3). This suggests that tuning CAR expression tunes perforin secretion, thereby tuning cytolytic function. While not equivalent to such effector functional assays, my transcriptomic findings on effector molecule genes corroborate these findings (Figure 5.3). Thus, tuning CAR expression appears as an attractive method to tune effector function.

As spotlighted in my introduction, Eyquem and colleagues notably optimised the level of CAR expression to a single, uniform level [99]. They accomplished this by using genome engineering technology CRISPR/Cas9 to introduce the CAR transgene into the T-cell receptor  $\alpha$  constant (*TRAC*) locus. This resulted in a T-cell receptor knockout and CAR knock-in substitute. This remarkably conferred uniform CAR expression; enhanced T cell potency in vivo; and mitigated tonic signalling,

effector T cell differentiation, and exhaustion. Their findings provided a robust functional basis for optimised CAR expression and the need for further investigations on the impact of CAR expression. However, their work only looked at two levels of expression. My doctoral work built upon their findings by vastly increasing the number of expression levels assessed and comprehensively dissecting the T cell transcriptomic response. In summary, my doctoral work provides a thorough molecular assessment of the impact of CAR expression in CAR-T therapies. I was able to demonstrate tuning of CAR expression to precise, step-wise levels, ultimately forming a noteworthy dynamic range. I paired this system with an in-depth transcriptomic assessment both at the bulk and at the single-cell level. In the following section, I will dive into some of my findings and suggest future steps in my study.

### 6.3 APPLICATIONS AND FUTURE DIRECTIONS

Chimeric antigen receptor T cell therapy has demonstrated remarkable success in addressing blood-based cancers. However, noteworthy limitations preclude the therapy from more widespread success. As discussed in my introduction, these include on-target, off-tumor toxicity, tonic signalling, and dysfunction, more commonly known as exhaustion. In this section, I highlight some of my noteworthy findings and suggest how tuned CAR expression may be able to address these outstanding roadblocks.

In my T cell model investigations, I demonstrated how tuning CAR expression controlled both the magnitude of activation and the proportion of cells activated (Figure 3.6). Toxic side effects, such as cytokine release syndrome (CRS) are observed in a majority of patients infused with CAR-Ts. Over-activation of CAR Ts has been implicated as a main cause for this, as well as the excessive secretion of cytokines when activated [104, 111, 126]. My findings (Figure 3.6) demonstrate a potential strategy to curtail and control activation, thereby reducing the likelihood of such debilitating toxic side effects.

Furthermore, I demonstrated how tuned CAR expression conferred tuned EC<sub>50</sub> values for a proxy T cell activation marker, NF- $\kappa$ B-EGFP in T cell models (Figure 3.6). This was demonstrated for both the magnitude of activation and the proportion of cells activated (Figure 3.6). These results suggest that tuning CAR expression accordingly tunes the threshold or amount of antigen required to elicit T

cell activation. Therefore, tuning CAR expression is an avenue to endow CAR-Ts with antigen-level discernment. CAR-T therapy has encountered numerous difficulties when attempting to translate into solid tumours. One challenge is to build CAR-T therapies that can discern between healthy cells and cancer cells. Indeed, for CARs targeting antigens that are expressed in both cell types, there is growing interest to prevent off-tumour, on-target T cell cytotoxicity. Often, CAR-targeted antigens on cancer cells are overexpressed. Thus, tuning CAR expression such that CAR-Ts only hone to cancer cells expressing high levels of target antigen is a promising avenue that can address this pressing issue.

Future *in vitro* and *in vivo* functional testing of my findings will be crucial before potentially translating miSFITs tuning system into current CAR-T therapies. For example, this could involve CAR-Ts tuned to 10 different levels and fluorescent target cells, akin to the experimental setup in Walker and colleagues' investigations [351]. Additionally, these target cells could be pulsed with different peptide concentrations. Subsequently, co-cultures of tuned CAR-Ts with equal proportions of each target cell line could be set up. Post incubation, flow cytometric measurement of fluorescence of each cell line would provide an assessment of antigen sensitivity and off-target cytotoxicity. Antibody staining for cell surface markers coupled with measuring cytokine secretion could provide valuable functional and proteomic measurements that could confirm and enhance my transcriptomic findings.

In another assay, I could harness my developed co-culture system with synthetic CHO-K1 target cells and introduce CAR-Ts of 10 levels of expression. After an incubation period, I could quantify CD3z phosphorylation, one of the key events in T cell activation and signalling, via intracellular staining as well as measure differentiation markers to assess differentiation rates, and couple this with measurements of cytokine secretion.

Tonic signalling is also another issue limiting CAR T therapy. It is the uncoordinated and constant activation of a CAR-T cell, demarcated by ligand-dependent or ligand-independent signalling [50, 60, 99, 141, 142]. In ligand-dependent signalling, targeted antigen persists, as observed in solid tumors and their microenvironment, thereby constantly binding and triggering CAR-mediated activation signals. In ligand-independent signalling, CARs spontaneously aggregate and cluster on the

T cell surface, resulting in separate CAR molecules interacting and binding with each other, eliciting activation signals [104, 141-144]. Tonic signalling often results in impaired T cell activation, effector function, and survival [142, 143] and is implicated in giving rise to T cell exhaustion. It is often characterised by constitutive phosphorylation of the CD3ζ intracellular domain [19, 50, 141, 143], secretion of excessive amounts of cytokines [104, 146], and accelerated T cell differentiation [147-149].

It has been suggested that CAR expression plays a key role in tonic signalling [141]. Indeed, Eyquem and colleagues demonstrated how optimised CAR expression abated tonic signalling and CAR-T exhaustion [99]. However, this remains to be comprehensively assessed. miSFITs robustly tune CAR expression transcriptionally and in surface protein (Chapters 3 in T cell models, 4 and 5 in primary T cells) using the miSFITs system. Thus, they represent an attractive avenue to thoroughly explore CAR expression's impact on tonic signalling.

T cell exhaustion, a key state defining T cell dysfunction, is another factor hamstringing the successful application of CAR-T therapies for solid tumours [21, 107, 108]. This has impacted T cell function by accelerating T cell differentiation and potentiating anti-tumour effects [19, 50, 99, 141, 143, 146, 148, 149, 169, 361-363].

My investigations in gene signatures of T cell dysfunction, activation, and memory revealed that CAR expression can be optimised to tackle the outstanding challenge of T cell exhaustion. More specifically, I observed a key inflection point in CAR expression that appeared to reduce exhaustion without sacrificing T cell activation and memory. This heavily suggests CAR expression can be tuned to this 'goldilocks zone' to give rise to exhaustion-resistant T cells. This would be a boon to addressing the longstanding challenge in CAR T therapy. However, this finding requires extensive of functional testing.

In addition, my mutual-information analysis shed light on another side of T cell dysfunction. More specifically, I observed genes of several key cellular processes, including cell division, metabolism, and RNA processing, becoming dysfunctional with unregulated CAR expression. The aforementioned 'goldilocks zone' could very well take into account these key cellular processes, striving for the least impairment.

A key experiment that would validate tuning CAR expression as a robust strategy would be assessing tuned CAR T cells' ability in mediating tumour growth. This could include conducting *in vivo* mice experiments in which CAR Ts of different expression levels are introduced and tumour size is monitored over an extended period. Additionally, I could extract any tumour infiltrating lymphocytes and measure T cell expansion indexes and expression patterns of inhibitory receptors, such as PD-1 and TIM3. Then, I could potentially identify the miSFIT that maximised tumour control and exhibited reduced T cell dysfunction. This would be a powerful finding that would clearly signal that tuning CAR expression should be employed as a means to overcome exhaustion.

#### 6.4 CONCLUDING REMARKS

In this thesis, I have documented my efforts to fine-tune chimeric antigen receptor expression and to dissect the subsequent T cell response. This assessment demonstrated robust tuning of CAR expression to step-wise levels, covering a broad dynamic range, achieving the first goal of this project. More importantly, this work fills a critical gap in knowledge within the cancer immunotherapy community. RNA sequencing allowed me to investigate the relationship between CAR levels and T cell gene expression profiles, accomplishing the second goal of this project. I envision the transcriptomic atlas against CAR expression provided here will be the basis for further investigations. Indeed, tuning CAR expression provides a promising avenue to address pressing, longstanding challenges hindering the success and widespread translation of CAR-T therapies.

# 7

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# Appendix

## A.1 TARGETS OF CHIMERIC ANTIGEN RECEPTORS

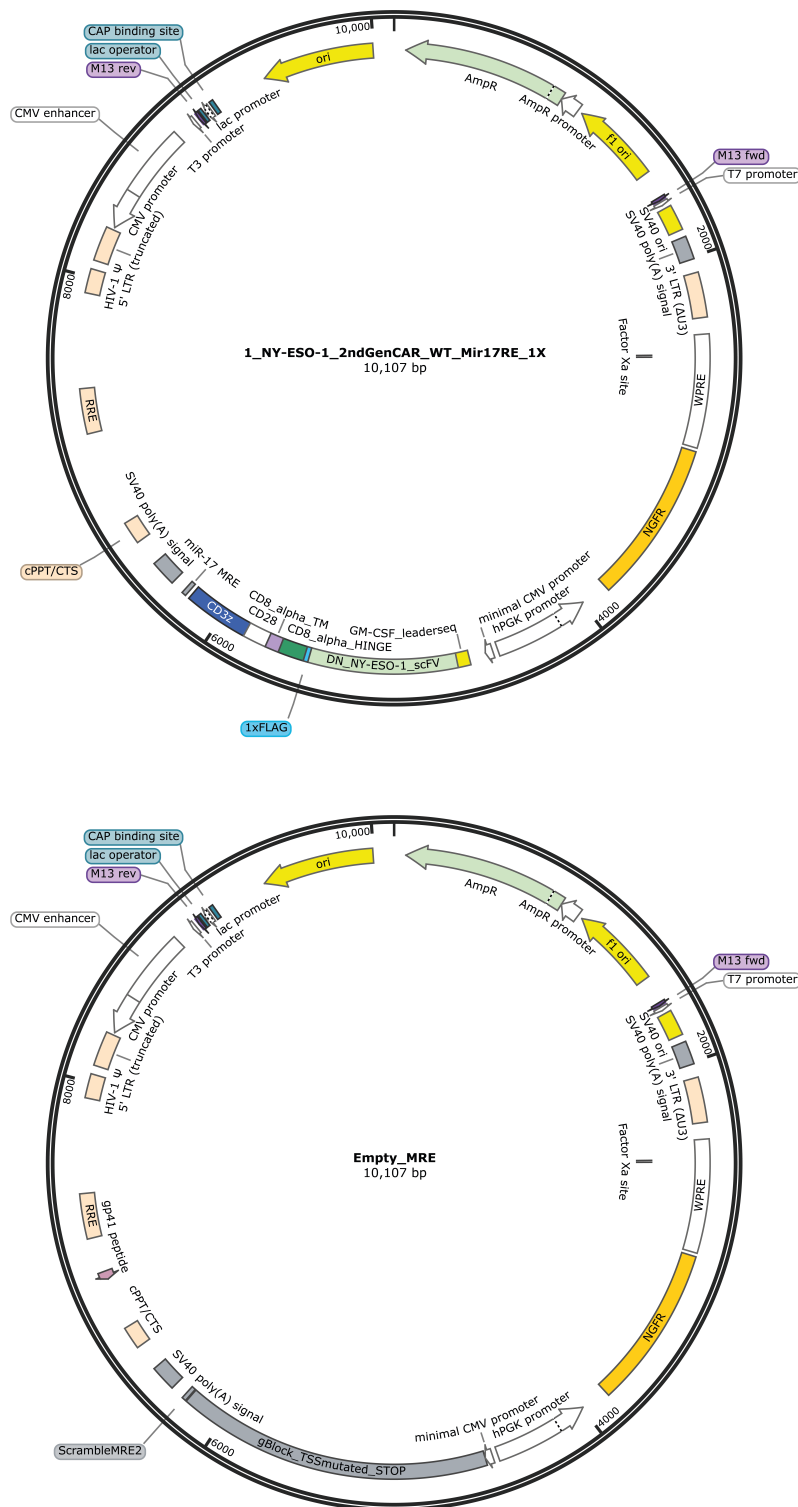
Included in this section is a table of the different antigens that have been targeted in CAR therapies alongside which tumours/cancers they have been detected expression in.

| Target ID         | Target Name                                             | Cancer/ Tumor                                                                                                                                              |
|-------------------|---------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------|
| AFP               | alpha-fetoprotein                                       | hepatocellular carcinoma, glioblastoma, pancreatic, breast, thoracic, mesenchymal tumors, osteosarcoma                                                     |
| ANTXR1            | anthrax toxin receptor 1                                | gastric tumors                                                                                                                                             |
| $\alpha V\beta_3$ | alpha V beta 3                                          | glioblastoma                                                                                                                                               |
| $\alpha V\beta_3$ | alpha V beta 6                                          | pancreatic, cervix, ovarian                                                                                                                                |
| AXL               | AXL receptor tyrosine kinase                            | renal cell carcinoma                                                                                                                                       |
| B7-H3             | B7 homolog 3 protein                                    | glioblastoma, ovarian                                                                                                                                      |
| B7-H6             | B7 homolog 6 protein                                    | gastrointestinal                                                                                                                                           |
| c-MET             | tyrosine-protein kinase Met                             | breast                                                                                                                                                     |
| CAIX              | Carbonic anhydrase IX                                   | renal cell carcinoma, glioblastoma                                                                                                                         |
| CD133             | prominin-1                                              | hepatocellular carcinoma                                                                                                                                   |
| CD147             | basigin                                                 | hepatocellular carcinoma                                                                                                                                   |
| CD171             | neural cell adhesion molecule L1                        | neuroblastoma                                                                                                                                              |
| CD32A             | Fc $\gamma$ RII                                         | lung                                                                                                                                                       |
| CD46              | membrane cofactor protein                               | colorectal                                                                                                                                                 |
| CD47              | integrin-associated protein                             | pancreatic, ovarian                                                                                                                                        |
| CD56              | neural cell adhesion molecule                           | brain cancer, neuroblastoma                                                                                                                                |
| CEA               | carcinoembryonic antigen                                | colorectal, pancreatic, thoracic, liver                                                                                                                    |
| CEACAM7           | carcinoembryonic antigen-related cell adhesion molecule | pancreatic                                                                                                                                                 |
| CLDN18.2          | Claudin 18.2                                            | pancreatic                                                                                                                                                 |
| CSPG4             | chondroitin sulfate proteoglycan-4                      | melanoma, glioblastoma, breast                                                                                                                             |
| DLL-3             | delta-like 3                                            | lung                                                                                                                                                       |
| DR5               | death receptor 5                                        | hepatoma                                                                                                                                                   |
| EGFR              | epidermal growth factor receptor                        | thoracic, mesenchymal tumors, osteosarcoma, lung, liver, stomach                                                                                           |
| EGFRvIII          | epidermal growth factor receptor variant III            | glioblastoma, lung                                                                                                                                         |
| EpCAM             | epithelial cell adhesion molecule                       | colorectal, prostate                                                                                                                                       |
| EphA2             | EPH receptor A2                                         | glioma                                                                                                                                                     |
| FAP               | familial adenomatous polyposis                          | Nectin4-positive advanced malignant solid tumor                                                                                                            |
| FR- $\alpha$      | folate receptor alpha                                   | ovarian; gastric tumors                                                                                                                                    |
| GD2               | Disialoganglioside                                      | glioblastoma, neuroblastoma, mesenchymal tumors, osteosarcoma, melanoma                                                                                    |
| gp100             | gp100                                                   | Melanoma                                                                                                                                                   |
| GPC2              | glypican-2                                              | neuroblastoma                                                                                                                                              |
| GPC3              | Glypican-3                                              | hepatocellular carcinoma; melanoma                                                                                                                         |
| GSPG4             | general secretion pathway protein G                     | melanoma                                                                                                                                                   |
| GUCY2C            | guanylate cyclase 2C                                    | Colorectal                                                                                                                                                 |
| HER2              | Human epidermal growth factor receptor 2                | head and neck, melanoma, glioblastoma, pancreatic, breast, thoracic, mesenchymal, osteosarcoma, central nervous system tumours, pediatric gliomas, gastric |
| IL-13R $\alpha$ 2 | Interleukin 13 receptor subunit alpha 2                 | glioblastoma                                                                                                                                               |
| L1-CAM            | L1 cell adhesion molecule                               | cervix                                                                                                                                                     |
| LMP1              | Epstein-Barr virus latent membrane protein 1            | nasopharyngeal                                                                                                                                             |
| MAGE-A1/3/4       | melanoma antigen gene protein                           | lung                                                                                                                                                       |
| MSLN              | Mesothelin                                              | glioblastoma, breast, thoracic, gastric, pancreatic, ovarian                                                                                               |
| MUC1              | mucin 1                                                 | pancreatic                                                                                                                                                 |
| MUC16             | mucin 16                                                | ovarian, thoracic, pancreatic                                                                                                                              |
| MUC1A             | mucin 3a                                                | gastric tumors                                                                                                                                             |
| NKG2D             | Natural killer group 2, member D receptor               | colorectal, gastric, pancreatic, glioblastoma, ovarian                                                                                                     |
| NY-ESO-1          | New York esophageal squamous cell carcinoma 1           | ovarian, melanoma, neuroblastoma, sarcoma, bladder, esophageal, hepatocellular, head and neck, ovarian, prostate, breast                                   |
| PAP               | prostatic acid phosphatase                              | prostate                                                                                                                                                   |
| PSA               | prostate-specific antigen                               | prostate                                                                                                                                                   |
| PSCA              | prostate stem cell antigen                              | pancreatic, prostate                                                                                                                                       |
| PSMA              | prostate-specific membrane antigen                      | prostate                                                                                                                                                   |
| ROR1/2            | RAR-related orphan receptors                            | thoracic, renal cell carcinoma                                                                                                                             |
| TAG-72            | tumour-associated glycoprotein 72                       | colorectal                                                                                                                                                 |
| TM4SF1            | transmembrane 4 L six family member 1                   | colorectal                                                                                                                                                 |
| VEGFR2            | vascular endothelial growth factor receptor 2           | melanoma, brain                                                                                                                                            |
| WT1               | Wilms tumor 1                                           | mesothelioma, gastrointestinal, glioblastoma, ovarian                                                                                                      |

**Table A.1:** Antigens targeted by Chimeric Antigen Receptor T therapy in pre-clinical and clinical studies.

## A.2 VECTORS USED FOR TUNING CHIMERIC ANTIGEN RECEPTOR EXPRESSION

Included in this section are representative examples of the expression vectors used in this study for the tuning of the DN-28z CAR with the miSFITs tuning system.



**Figure A.1:** Representative expression vectors used for tuning chimeric antigen receptor expression

### A.3 GENE SIGNATURE HEATMAPS

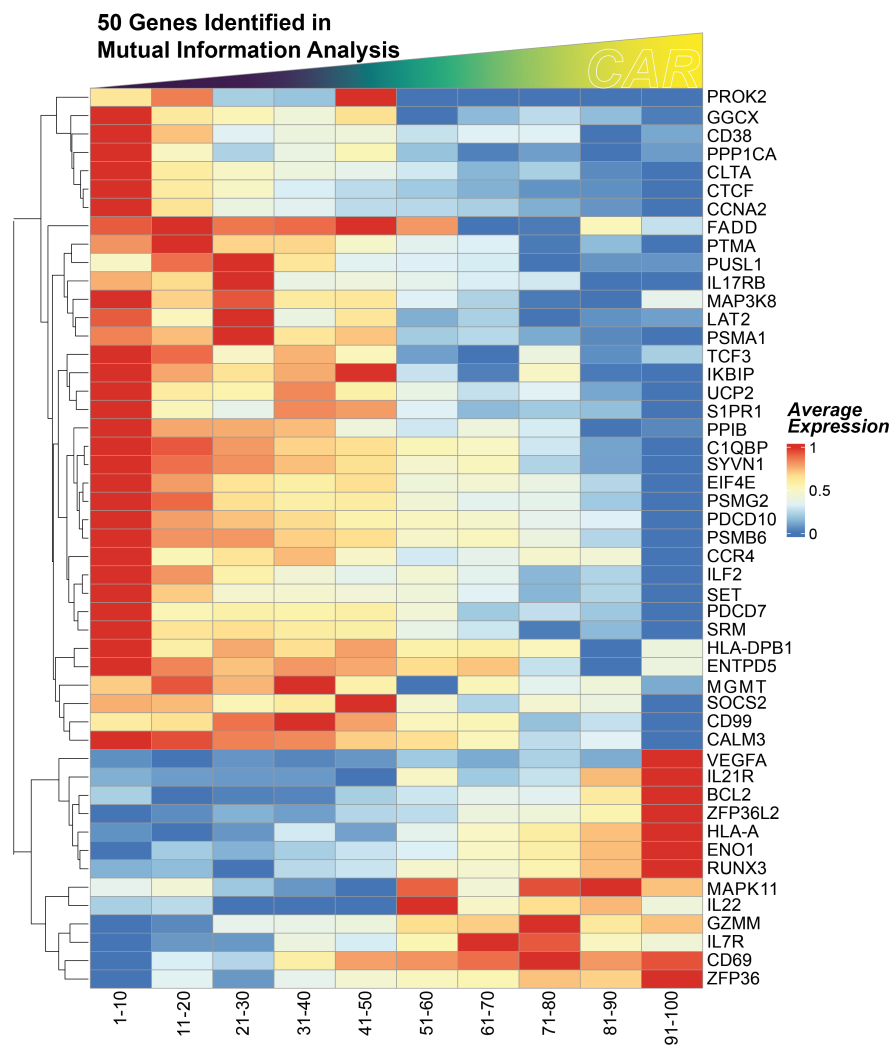
Included in this section are heatmaps depicting the component genes used in gene signature analysis.



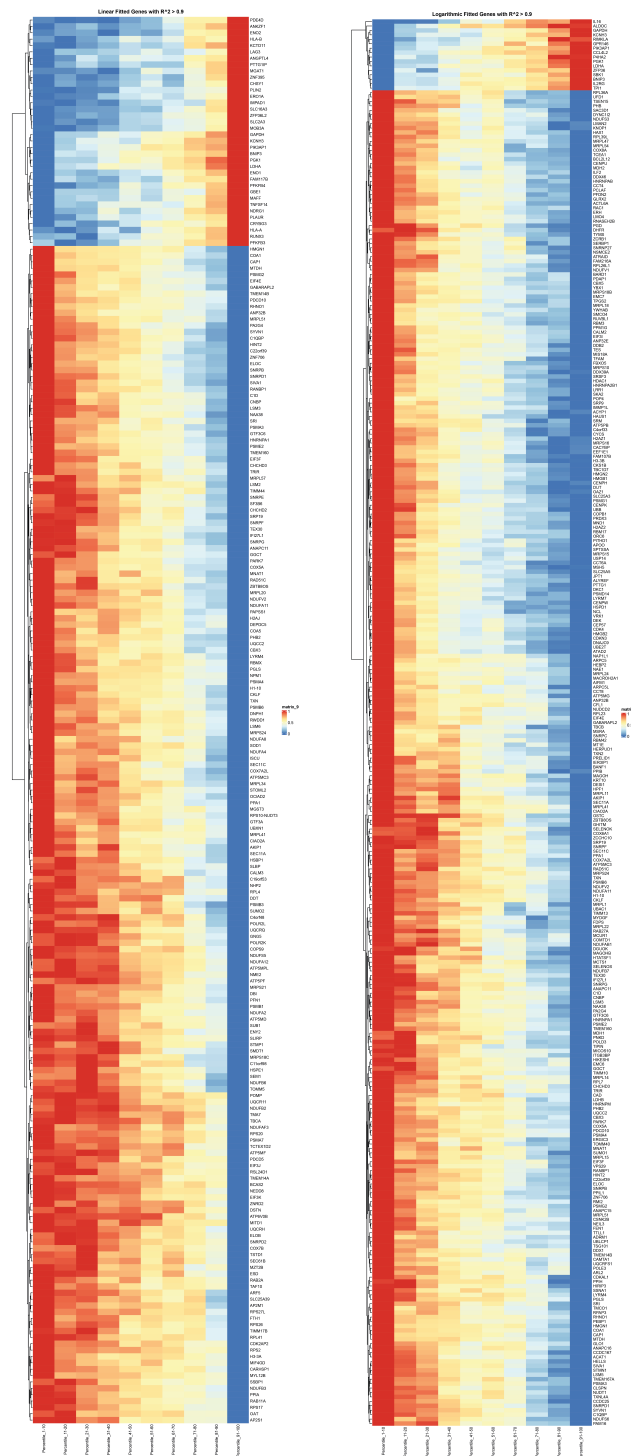


#### A.4 UNBIASED DISCOVERY HEATMAPS

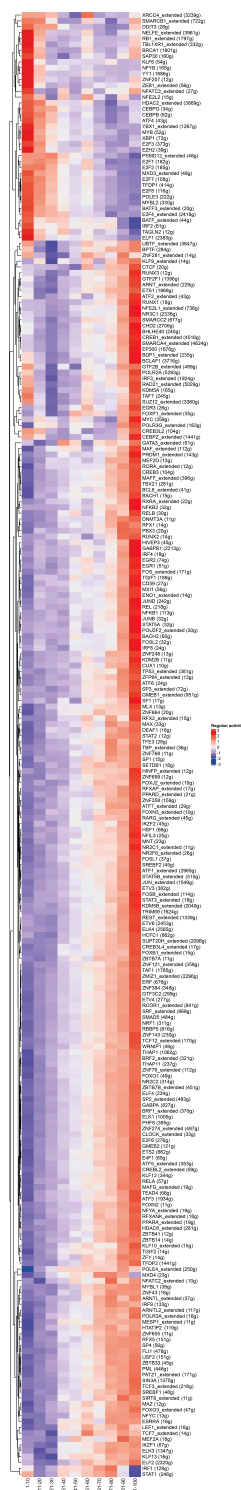
Included in this section are heatmaps of all genes and regulons identified in my unbiased discovery analyses.



**Figure A.3:** Heatmap depicting 50 genes identified in mutual information analysis. Genes included have a normalised mutual information of 1 with DN-28z CAR expression.



**Figure A.4:** Heatmap depicting all genes identified in linear and logarithmic regression fitting analysis (Figure 5.8). Genes included were identified to have a  $R^2 > 0.9$  with DN-28z CAR expression



## A.5 OLIGONUCLEOTIDES USED IN THIS STUDY

Included in this section is a table depicting the oligonucleotides used in this study. All oligonucleotides were synthesized by Integrated DNA Technologies.

| Oligo Name         | Oligo Sequence                                                     |
|--------------------|--------------------------------------------------------------------|
| DN28zCAR-F         | GGAGCTCCTGCAGGTGCCGCCATGCTGCTGctggtcaca                            |
| DN28zCAR-R         | GGTAGGCTAGCACTCACGTGCTACCCCTTATCTTGGAGGCAGGGCC                     |
| LentiBB-CMV-seq    | TCGTTTAGTGAACCGTCAGATC                                             |
| LentiBB-SV40pA-seq | CGATCACGAGACTAGCCTCGAG                                             |
| bi-dir-Miseq-F     | ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGGAAGAATCCTCAAGAGGGC             |
| bi-dir-Miseq-R     | GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCCCCGGTGCATGACTAAG              |
| J-CARgTransd-F     | CGCCTACCGGTCCAGAGTGA                                               |
| J-CARgTransd-R     | CGATGACAGGCGCAGCTTCC                                               |
| J-CARgTransd-seq   | GCTGCAGAAAGACAAGATGGC                                              |
| 10X-CARmiSFIT-F    | CTACACGACGCTCTTCCG                                                 |
| 10X-CARmiSFIT-F    | TGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCAAGATGGCCGAAGCCTACA              |
| TruSeq-501-10XCAR  | AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCG |
| TruSeq-701-10XCAR  | CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGT              |
| 10x-EmptymiSFIT-F  | TGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGTGAGACTCGGCTGgcta                |
| 10x-EmptymiSFIT-R  | CTACACGACGCTCTTCCGATCT                                             |
| TruSeq-501         | AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGAC          |
| TruSeq-701         | CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGT              |
| Ad1-noMX           | AATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCGTCAGATGTG                 |
| Ad2.1-TAAGCGCA     | CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGGAGATGT              |
| Ad2.2-CGTACTAG     | CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGGAGATGT              |
| Ad2.4-TCCTGAGC     | CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGGAGATGT              |

**Table A.2:** Oligonucleotides used in this study

## A.6 FLOW CYTOMETRY REAGENTS USED IN THIS STUDY

Included in this section is a table of the flow cytometry reagents used for this study.

| Reagent                  | Fluorophore | Supplier        | Clone    | Catalogue number |
|--------------------------|-------------|-----------------|----------|------------------|
| NGFR (CD271)             | V450        | BD              | C40-1457 | 562123           |
| FLAG (DYKDDDDK)          | APC         | Miltenyi Biotec | REA216   | 130-119-584      |
| CD45                     | BV421       | BioLegend       | 2D1      | 368521           |
| CD69                     | AF547       | BioLegend       | FN50     | 310918           |
| Zombie Green             | None        | BioLegend       | N/A      | 423111           |
| Human BD Fc Block        | None        | BioLegend       | N/A      | 564220           |
| Precision counting beads | None        | BioLegend       | N/A      | 424902           |

**Table A.3:** Flow cytometry reagents used in this study