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UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

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Ex vivo manipulation of CD8 T cells to improve adoptive cell therapy against cancer

By

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Submission of PhD thesis

.

Abstract

Adoptive cell transfer (ACT) is a potentially curative cancer therapy in which autologous tumour-specific lymphocytes undergo vast levels of *ex vivo* expansion before being reinfused into the patient. Despite many promising results, ACT efficacy is limited by both poor persistence and weak anti-tumour responses, mainly due to high levels of terminal differentiation displayed by transferred T cell and immunosuppressive factors associated with the tumour micro-environment. Technical developments in genetic modifications have allowed for the manipulation of T cell longevity and function, improving the potency of ACT and broadening the range of treatable cancers. The investigation of which genetic modifications are best suited for enhancing the function of CD8 T cells in the context of ACT is therefore a growing area of research. Here, I developed a protocol using OT-I T cells for the treatment of mice bearing EG7 tumours, including vaccination and sublethal-irradiation strategies, where the impact of genetic modifications could be assessed. Firstly, I aimed to enhance the formation of memory CD8 T cells, as this phenotype is positively correlated with improved ACT therapy in clinical and preclinical settings. Here, the overexpression of either FOXO1, FOXO3a, or Eomes was sufficient to drive cells towards aspects of the classic memory profile, yet this did not lead to improved responsiveness during *in vivo* vaccination of immune competent mice. While enhanced engraftment was achieved by T cells expressing constitutively active FOXO1 in a lymphopenic setting, these cells had limited peripheral surveillance and showed reduced anti-tumour immunity in an ACT model. These data highlighted the complexities of interpreting the phenotype of cells in relation to function while overexpressing transcription factors. Following this work, I aimed to improve the sensitivity of CD8 T cell stimulation through the knockdown of phosphatases or a kinase that are known to limit aspects of TCR signalling. With a mostly unaltered phenotype following the IL-2 mediated expansion, the *in vitro* restimulation of transduced CD8 T cells demonstrated that the knockdown of PTPN2, PTPN22, or CSK enhanced IL-2 expression in response to TCR stimulation. However, neither cytotoxic molecule production nor ability to kill target cells was observed *in vitro*. *In vivo,* it was shown that PTPN2 knockdown uniquely improved CD8 T cell accumulation and granzyme B expression in response to vaccination while also mediating a positive bystander effect for non-transduced cells. As such the knockdown of PTPN2 granted improved anti-tumour immunity within a preclinical ACT model, accompanied by substantial increased in the accumulation of T cells *in vivo*. The work here highlights the benefits of disrupting factors that suppress T cell stimulation and opens the possibility of future work utilising PTPN2 knockdown alone or in combination with other therapeutic strategies to augment the efficacy of ACT.

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List of abbreviations

ACT	Adoptive cell transfer
Akt	Protein kinase B
APC	Antigen-presenting cell
BCR	B cell receptor
BLIMP	B lymphocyte-induced maturation protein-1
CAFs	Cancer-associated fibroblast
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CDR	Complementarity determining region
CEA	Carcinoembryonic antigen
CEA	Carcinoembryonic antigen
ConA	Concanavalin A
CSK	C-terminal Src kinase
CTLA4	Cytotoxic T-Lymphocyte Associated Protein 4
CTV	Celltrace violet
DAG	Diacylglycerol
DC	Dendritic Cell
DN	Double negative
DP	Double positive
ER	Endoplasmic reticulum
ERAP1	ER aminopeptidase 1
FCS	Fetal calf serum
FOX	Forehead box
GzmB	Granzyme B
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
i.v.	Intravenous
ID	Inhibitor of DNA binding
IDO	Indoleamine 2,3-dioxygenase

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IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IP3	Inositol 1,4,5-trisphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
KLRG1	Killer-cell lectin like receptor G1
LCMV	Lymphocytic choriomeningitis virus
LM	Listeria monocytogenes
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinases
M-CFS	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MPEC	Memory precursor effector cell
mTOR	Mechanistic target of rapamycin
NK	Natural killer
OVA	Ovalbumin
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
Pip2	Phosphatidylinositol (3,4)-bisphosphate
Pip3	Phosphatidylinositol (3,4,5)-trisphosphate
PKCθ	protein kinase Cθ
PLC	Phospholipase C
pMHC	Peptide-bound MHC
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
PTPN	Protein tyrosine phosphatase non-receptor

RHEB	Ras homolog enriched in brain
RHEB	Ras homolog enriched in brain
Ser	Serine
SIIN	SIINFEKL
SIIQ	SIIQFEKL
SIIT	SIITFEKL
SLEC	Short-lived effector cells
SNP	Single nucleotide polymorphism
ssRNA	Single-stranded RNA
STAT	Signal transducers and activators of transcription
TAA	Tumour-associated antigen
TdT	Terminal deoxynucleotidyl transferase
Thr	Threonine
TLR	Toll-like receptor
Tyr	Tyrosine

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Academic Thesis: Declaration Of Authorship

I, Henry Leonard, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

'Ex vivo manipulation of CD8 T cells to improve adoptive cell therapy against cancer'

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
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5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
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Acknowledgements

For your invaluable guidance, patience, and wisdom, Aymen and Anne, thank you.

Chapter 1: Introduction

1.1 The immune system

The immune system comprises a broad range of cells and processes that defend the host against certain diseases. In humans, innate and adaptive responses exist, allowing for the recognition and elimination of infectious pathogens as well as cancerous cells. In the past few decades, the immune system's ability to target and destroy tumours has been exploited through the development of novel immunotherapies for the treatment of cancer.

1.1.1 The innate immune system

The innate immune system is characterised by an immediate non-specific response against pathogens through evolutionarily conserved mechanisms. While discriminating self from non-self, a variety of innate immune cells, including natural killer (NK) cells, macrophages, neutrophils, eosinophils, and dendritic cells (DCs), have specialised functions to counter disease [1].

DCs are professional antigen-presenting cells (APCs) that bridge the gap between innate and adaptive immunity. Subsets of DCs, including $CD11b^+$, $CD103^+$, and Langerhans cells, circulate in the blood and patrols peripheral tissues such as the skin, lungs, and intestines, which are regularly exposed to pathogenic threats. Within the peripheral sites, DCs sample exogenous proteins through phagocytosis or endocytosis then present antigens via major histocompatibility complex (MHC) class II molecules to CD4 T cells or MHC class I molecules to CD8 T cells. DCs also migrate to the sites of inflammation and mature following the recognition of common pathogen motifs with Toll-like receptors (TLRs) [1]. Importantly, mature DCs can migrate to secondary lymphoid organs, where antigens are presented alongside appropriate costimulatory molecules to initiate an adaptive immune response. Although other APCs can present antigens to CD8 T cells, DCs are the greatest contributor to this process [2].

1.1.2 The adaptive immune system

The adaptive immune system consists of lymphocytes, T cells and B cells, which possess specialised antigen receptors and can form long-lasting memory subsets that persist for years or even decades [3]. The antigen receptors on lymphocytes achieve a vast level of diversity through the genetic recombination of T cell receptor (TCR) and B cell receptor (BCR) genes. While lymphocytes are initially slow to respond to pathogens, relative to innate responses, once activated these cells can mediate effective elimination of cells displaying the target antigen [4]. Furthermore, the persistence of specialised memory lymphocytes can provide very effective

protection upon re-exposure to an antigen, due to the higher number of antigen-specific cells and fast response time compared to naive cells [5]. For T cells, two main groups can be distinguished based on the expression of the CD4 or CD8 co-receptor. Conventional CD4 T cells, known as the T helper (T_h) lineage, express high levels of cytokines to support CD8 T cells and B cells, although some CD4 T cell subsets can also possess cytotoxic capabilities [6]. CD4 T cells are activated by antigenic peptides presented by MHC class II molecules, mainly expressed by APCs [7]. There is also a regulatory arm of the CD4 T cell lineage known as T_{regs}, which suppress the immune system and maintains tolerance towards self-peptides. CD8 T cells are characterised by their ability to kill cells that display antigenic peptides bound to MHC class I molecules.

1.1.3 MHC class I antigen processing and presentation

MHC class I molecules are expressed by almost all nucleated cells, allowing for specific recognition and destruction of infected or abhorrent cells by CD8 T cells. Classically, endogenous proteins are processed to provide peptides, which are then loaded into the binding groove of an MHC class I molecule and presented as antigens on the surface of the cell. As the peptides produced from foreign or mutated proteins are classed as not self-antigens, these can provide immunogenic targets for a TCR. The processing and presentation of antigens is a crucial process in cellular immunity. Intracellular proteins are continuously being broken down and recycled by proteasomal degradation, generating short fragments of amino acids that can enter antigen-processing pathways [8, 9]. The transporter associated with antigen processing (TAP) facilitates the ingress of antigens into the rough endoplasmic reticulum (ER). The antigens can then be modulated by enzymes, such as ER aminopeptidase 1 (ERAP1) before typically an 8-10 amino acid fragment binds to the groove of an MHC class I molecule and is transported to the cell surface via the Golgi apparatus. The non-classical system of MHC class I presentation can utilise exogenous proteins that are endocytosed before being degraded by lysosomal proteases. This cross-presentation pathway is extremely important, as it can provide the initial priming stimulus to CD8 T cells from APCs that have acquired exogenously derived proteins from tumour cells [10].

1.2 CD8 T cells

CD8 T cells are highly specialised lymphocytes that can detect and destroy infected or cancerous cells. For this report, the term CD8 T cell will refer to lymphocytes possessing the αβTCR, constituting 90% of the total CD8 T cell population, which enables the recognition of peptides bound to MHC class I molecules. Understanding the mechanism of TCR/MHC interactions and subsequent signalling guiding CD8 T cell function is important for furthering the development of immunotherapies.

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1.2.1 CD8 T cell development

Hematopoietic stem cells (HSCs), derived from the red bone marrow's mesoderm cells, can give rise to myeloid and lymphoid lineages. The HSC progenitors that migrate to the thymus, termed thymocytes, undergo a series of maturation steps, which can ultimately result in the formation of αβ T cells [11]. At the earliest stage of development, double negative (DN) thymocytes express neither the CD4 nor CD8 co-receptor. Further classification of maturation status is achieved through expression of the surface markers CD25 (IL-2Rα) and CD44 (an adhesion molecule). During the DN3 stage, $CD44^-$ $CD25^+$ thymocytes undergo β-selection, during which variable (V), diversity (D) and joining (J) gene recombination occurs, producing a highly specific pre-TCR [12]. Firstly, the D to J recombination of the βTCR chain occurs through either the joining of a Dβ1 gene to one of six Jβ1 segments or the joining of the Dβ2 gene to one of six Jβ2 segments. Following the DJ recombination, a Vβ segment is added, and sections between the segments are deleted. Variability in the joints between segments, resulting from the removal of nucleotides and the random addition of nucleotides by terminal deoxynucleotidyl transferase (TdT), further enhances TCR diversity [13]. The transcribed VDJ gene incorporates a constant gene to form TCR Cβ. The DN4 stage, $CD44^-$ $CD25^-$, is followed by αTCR chain recombination, which involves the same process as βTCR recombination with the V and J chains only. The assembly of the full-length TCR Cα and TCR Cβ forms the αβTCR, which is accompanied by the expression of both CD4 and CD8 co-receptors to form a double positive (DP) thymocyte.

It is a requirement that all DP cells interact with self-antigen presented on MHC class I or MHC class II molecules with an appropriate affinity in order to survive. In this positive selection process, approximately half of the DP thymocytes that express weak affinity TCR die by apoptosis due to a lack of survival signals [14]. The DP cells migrating to the medulla of the thymus then interact with self-antigen presented by APCs. This negative selection stage results in approximately 50% of the DP cells undergoing BIM-mediated apoptosis due to high-affinity TCR interaction with self-antigen. After positive and negative selection, the co-receptor that was not initially stimulated is downregulated, leaving either a single positive CD4 T cell or CD8 T cell [14]. These naive cells can then exit the thymus and migrate to secondary lymphoid organs.

Although positive selection ensures the TCR repertoire is rendered towards MHC reactivity, it is contentious whether the negative selection process reduces the risk of autoimmunity. For example, far fewer cells die during negative selection for $BIM^{-/-}$ mice, which therefore possess a larger repertoire of self-reactive T cell clones, yet these mice do not suffer from widespread

autoimmunity [15]. Additionally, there are no autoimmune diseases for which the pathology has been shown to be a direct result of disrupting clonal deletion [15]. This highlights the power of peripheral tolerance mechanisms to block the activity of self-reactive T cells. A theory by Manson postulates that in fact, all T cells must be extremely cross-reactive [16]. Assuming that 1% of peptides can bind to a given MHC molecule a 10 amino acids chain can amount to the presentation of $1x10^{11}$ different combinations, yet estimates place the number of distinct TCRs in the human naive T cell pool at around 10^8 [17]. Comprehensive cover of potential foreign antigens must therefore be achieved by allowing TCRs to recognise multiple targets at varying affinities. Indeed, studies have shown that T cells can be activated by peptides that are unrelated to the sequence they were selected against [18, 19]. As such, future therapeutic interventions stimulating or manipulating TCRs must consider the risks of cross-reactive T cells overcoming tolerance and mediating off-target immunity.

1.2.2 CD8 T cell stimulation

Naive T cells preferentially migrate from the blood to secondary lymphoid organs, such as the spleen and lymph nodes, where they interact with APCs to become activated. This process occurs in the paracortex region of the lymph nodes or the white pulp surrounding the splenic arteries. While TCR signalling results from interactions with peptide-bound MHC (pMHC) class I, costimulation is also required for full activation of CD8 T cells. Mature DCs are crucial APCs, as they express high levels of MHC class I molecules as well as costimulatory molecules to enable an effective T cell stimulation [20]. As a T cell enters a lymph node, it migrates randomly, scanning many DCs until it binds with a high affinity to a pMHC complex. The initial scanning process is antigen-independent, facilitated by adhesion molecules such as grabbing integrin (DC-SIGN) and ICAM-3, expressed by DCs and T cells respectively. Additional stabilisation is achieved by other adhesion integrins such as LFA-1, which enable consistent signalling between pMHC and the TCR complex [21]. The activation of CD8 T cells can be aided by CD4 T cells directly, through the expression of costimulatory ligands, and indirectly by the secretion of cytokines. For example, IL-2 promotes the proliferation of CD8 T cells and upregulates costimulatory molecules on DCs. Interestingly, for some infection models, CD8 T cells primed in the absence of CD4 T cells can be far less effective at mounting primary immune responses [22-24]. Classically, the stimulation of T cells is broken down into three signal groups, signal 1 provided by TCR, signal 2 provided by costimulation, and signal 3 provided by cytokines. Each of these components is important in shaping the activation and differentiation of CD8 T cells.

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1.2.3 Signal 1: TCR signalling

The α and β TCR chains form a complex with CD3 chains to comprise the TCR, classified as type 1 transmembrane dimeric protein within the immunoglobulin superfamily. The TCR is composed of a membrane proximal constant domain and a membrane distal variable domain that has three complementary-determining regions (CDRs). This structure endows high levels of TCR specificity for the recognition of peptides presented by MHC molecules. TCR signalling is dependent on the kinase activity of Src family kinases (SFKs), as it lacks intrinsic enzymatic activity to initiate signalling (**Figure 1.1**). The binding of Lck to the CD8 co-receptor facilitates the phosphorylation of immunoreceptor tyrosine-based activation motif (ITAMs) substrates on the TCR-associated CD3 chains [25]. The phosphorylation of these ITAMs allows for the recruitment of ζ chain associated protein kinase (Zap70), which is also phosphorylated by Lck [26]. The activation of Zap-70 is a crucial step in TCR signalling as it phosphorylates adaptor proteins such as linker for activation of T cells (LAT) and SLP-76, which regulate many aspects of T cell function. For instance, the complex formed by these adaptor proteins, termed the LAT signalosome, facilitates the activation of Phospholipase C (PLC)γ1, which catalyses Phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 activates calmodulin through the release of calcium ions from the endoplasmic reticulum, promoting IL-2 production through NFAT activity. DAG activates protein kinase Cθ (PKCθ) to promote NF-κb activity, while also activating AP-1 through the Ras/mitogen-activated protein kinase (MAPK) pathway. TCR-induced activation of the LAT signalosome also reduces the mobility of T cells via changes in the cytoskeleton through polymerisation of actin, allowing robust contact between pMHC and TCR. In addition to these effects, TCR signalling cascades onto the Phosphatidylinositol-4,5-bisphosphate 3-kinase/ protein kinase B (PI3K/Akt) pathway, regulating many aspects of T cell differentiation through the control of transcription factors such as eomesodermin (Eomes) and Forkhead box (FOX)O1.

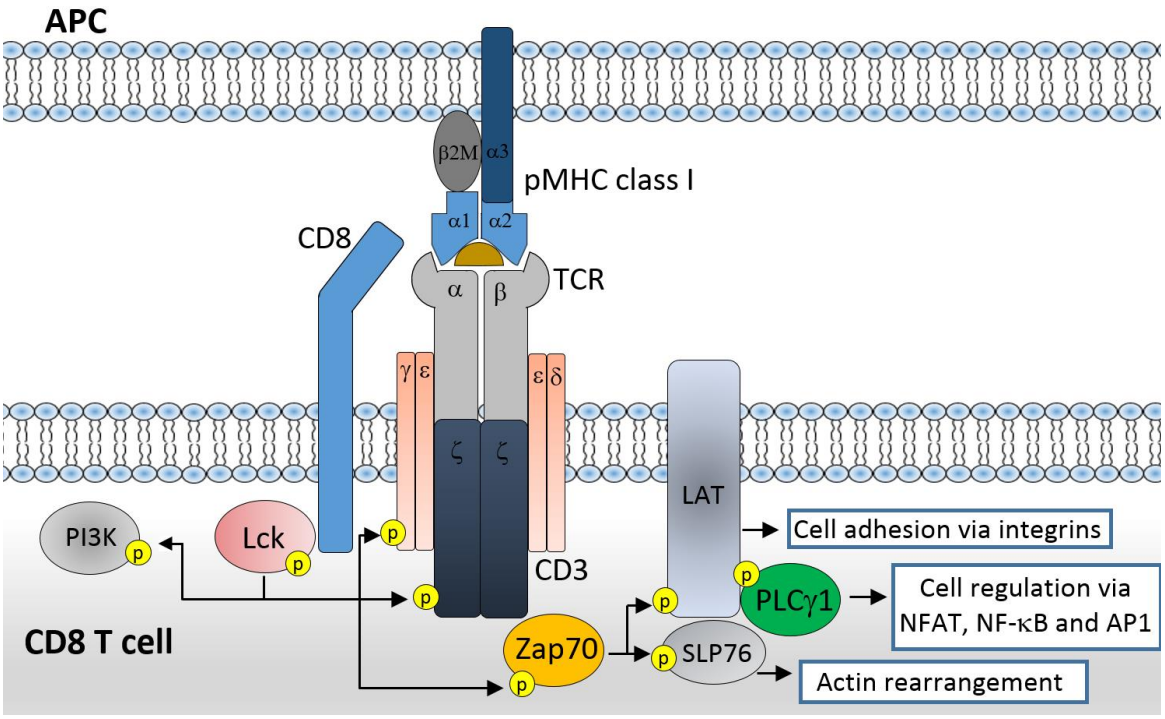

Figure 1.1: An overview of TCR signalling for CD8 T cells following pMHC interaction. The TCR complex is comprised of α and βTCR chains associated with various CD3 chains (δ,ε,γ, and ζ). Following a TCR interaction with a pMHC, Lck co-localises with the TCR complex, where it can phosphorylate ITAMs on CD3 chains. The resulting conformational changes allow Zap70 to bind to the CD3 ζ chain, where it is phosphorylated by Lck. Phosphorylated Zap70 transduces signals to downstream substrates, such as LAT and SLP76, resulting in the formation of a LAT signalosome. Signals cascading from the LAT signalosome control many aspects of CD8 T cell activation. Furthermore, TCR signalling cascades down to the PI3K/Akt pathway regulating function and differentiation of CD8 T cells.

1.2.4 Signal 2: Costimulatory signalling

Under normal circumstances, TCR signalling alone is not sufficient to activate naive CD8 T cells. Indeed, sustained signal 1 stimulation in the absence of costimulation can drive T cells to towards anergy and apoptosis [27]. Costimulation, termed signal 2, is required alongside signal 1 for optimal proliferation, survival, and differentiation of CD8 T cells, thereby distinguishing activation from anergy [25]. As such, the expression of costimulatory ligands on APCs guides the functional response of CD8 T cells. Costimulatory molecules can be classified within the superfamily of immunoglobulin (Ig) receptors/ligands such as CD28/B7.1-B7.2, CD2/LFA-3, and ICOS/ICOSL, and the tumour necrosis factor (TNF) receptor/ligand superfamily such as CD27/CD70, OX40/OX40L, and 4-1BB/4-1BBL (**Figure 1.2**). A well-studied example of costimulation comes from CD28 interaction with B7.1 (CD80) and B7.2 (CD86), providing activatory signals during T cell stimulation. The homodimeric glycoprotein CD28 is expressed on subsets of CD8 T cells, while its ligands are expressed on activated APCs. The activation of CD28 results in the activation of NF-κb

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and AP-1, promoting proliferation, cytokine production, cytoskeletal rearrangement, and survival through the upregulation of the anti-apoptotic factor Bcl-X_L [28]. CD28 can also cluster alongside the TCR, which sustains the immune synapse and aids in the recruitment of PKCθ. While CD28 is not a prerequisite for T cell activation, its presences can substantially lower the antigen threshold required for T cell activation, by approximately 7-fold, illustrating potent synergy between TCR and costimulatory signals [29].

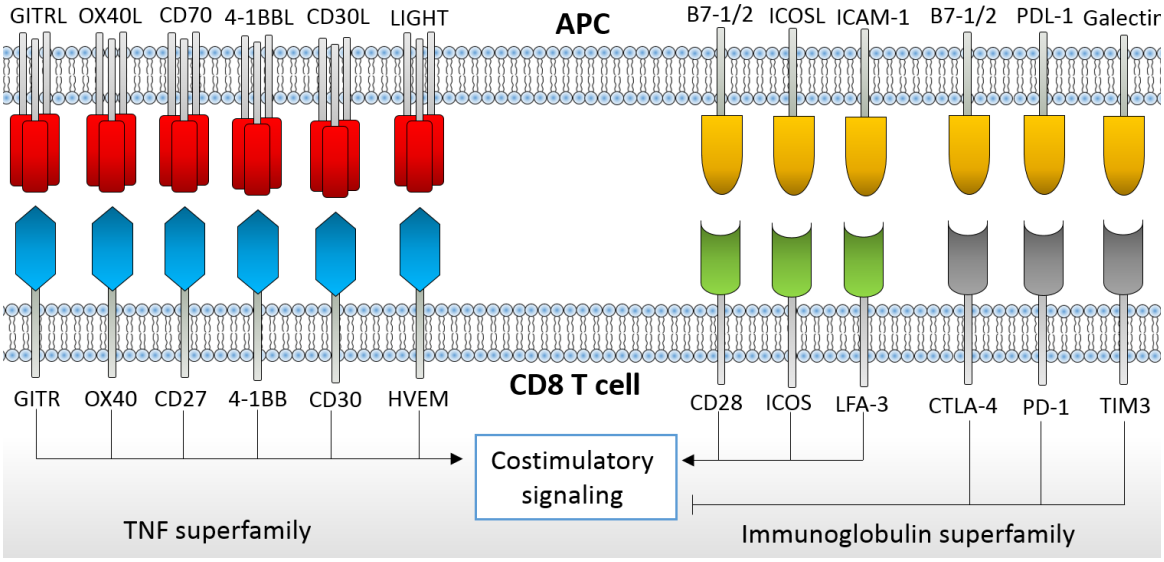

Figure 1.2: Cosignalling from the TNF and Ig superfamily in CD8 T cells. Stimulation of TNF superfamily or the Ig superfamily provides cosignalls that govern CD8 T cell function. The Ig family includes the costimulatory receptors (green) that provide positive signals to enhance T cell activation, while the coinhibitory receptors (grey) function to suppress CD8 T cell stimulation.

Cosignalling can be dramatically influenced by suppressive signalling molecules that block the stimulation of T cells. The antagonism of CD28 signalling by cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) is an example of checkpoint molecules preventing activation of CD8 T cells. CTLA-4 is a homolog of CD28 that does not transduce activatory signalling but binds to B7 ligands with a 20-fold higher affinity compared to CD28. The expression of CTLA-4, on T_{regs} and CD8 T cells, vastly outcompetes CD28 signalling, thereby preventing costimulation and promoting tolerance [30]. As well as CTLA-4, many other receptors can limit T cell activation and survival by transducing intracellular signals to limit CD8 T cell response to stimulation, including PD-1, TIM3, and LAG3. The expression of these factors can be associated with an exhaustion phenotype, in which CD8 T cells become unresponsive to stimulation [31].

1.2.5 Signal 3: Cytokine signalling

CD8 T cell activation is also influenced by cytokine stimulation, termed signal 3 [25]. As well as regulating the innate immune system, cytokines contribute to the regulation of effector function, survival, proliferation, and differentiation of CD8 T cells. Proinflammatory cytokines, such as type I interferons (IFNs), interleukin (IL)-1, IL-2, IL-6, and IL-12, provide stimulus to enhance activation levels. For example, the presence of IL-12 or IFNγ can promote the expansion of effector CD8 T cells [32]. As such, defects in either IL-12R or IFNR in CD8 T cells are severely detrimental to the formation of effector cells during inflammatory infections, as shown in *Lymphocytic choriomeningitis* virus (LCMV) and *Listeria monocytogenes* (LM) models [33-35]. IL-2 also contributes to the regulation of CD8 T cell fate. Expressed by NK cells, DCs, and activated T cells, IL-2 signals to CD8 T cells through a high-affinity receptor formed from common gamma chain (γc) (CD132), IL-2Rα (CD25), and IL-2Rβ (CD122). The stimulation of the IL-2R results in the phosphorylation of STAT5 and activation of the PI3K pathway, enhancing CD8 T cell proliferation.

Certain anti-inflammatory cytokines, such as IL-10 and TGFβ secreted by suppressive cells such as T_{reg} cells, limit CD8 T cell activation and survival. The binding of TGFβ to its receptor on CD8 T cells mediates the phosphorylation of Smads, causing a reduction in proliferation, cytotoxicity, and survival [36]. While inhibitory factors are important in preventing autoimmune disease, they often impede the anti-tumour response by CD8 T cells in the context of the tumour microenvironment.

1.2.6 CD8 T cell effector function

CD8 T cell function depends on the balanced of activatory and inhibitory signals. However, appropriate TCR stimulation alongside costimulatory molecules and cytokines that promote CD8 T cell activation will result in the rapid expansion and acquisition of effector function. This coincides with reduced expression of lymphoid homing markers, such as CD62L, and upregulation of chemokine receptors, including CCR2, CCR5, and CXCR3, which promote the migration of T cells to peripheral tissues and sites of inflammation. Without the need for further costimulation, activated CD8 T cells can recognise and destroy infected or cancerous cells that express the target antigen. CD8 T cells release granzymes and perforin molecules, which are packaged in cytotoxic granules, to kill target cells. Perforin binds to the target cell's membrane to form a pore, after Ca^{2+} dependent oligomerization, through which granzymes can enter. Granzyme B induces apoptosis using a caspase 3 dependent mechanism, as well as other caspase-independent mechanisms [37, 38]. While producing cytotoxic molecules and secreting cytokines such as IFNγ and TNFα to

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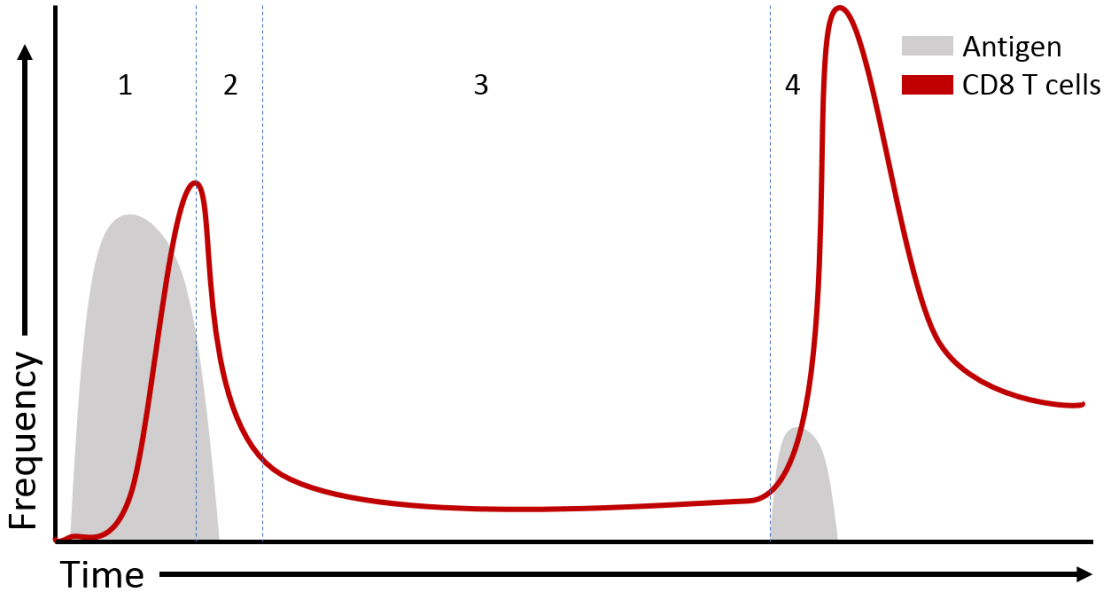
further stimulate the immune response, CD8 T cells can also selectively kill target cells expressing Fas ligands. Interaction of Fas with its ligand results in the activation of caspase-8, which subsequently results in the release of pro-apoptotic proteins such as cytochrome c [39].

Providing primary expansion of CD8 T cells is sufficient to clear the antigen, the contraction phase is entered (**Figure 1.3**), whereby approximately 90-95% of activated cells undergo apoptosis through one of several mechanisms [40]. Cytokine deprivation termed activated T cell autonomous death (ACAD), can lead to the upregulation of mitochondrial apoptotic factors. Alternatively, interactions with the TNFRs of neighbouring cells, such as CD30, can cause activation-induced cell death (AICD). The CD8 T cells that survive the contraction phase form the memory population and can persist for many years in an antigen-independent context, with homeostatic proliferation mediated by cytokines such as IL-15 [41]. These memory cells can provide long-lived protection against disease, rapidly regaining effector function and proliferating upon re-exposure to the target antigen.



1: Primary expansion in response to antigen encounter
2: Contraction following antigen clearance
3: Cytokine mediated survival of memory cells
4: Secondary expansion following antigen re-encounter

Figure 1.3: CD8 T cell response to antigen encounter and re-exposure. Naïve CD8 T cells are activated following an interaction with cognate antigen presented alongside appropriate costimulation and cytokines stimulation. 1) This stimulation causes the T cell to divide rapidly and gain effector functions, forming the primary response. 2) Once the antigen is cleared, the contraction phase is entered whereby approximately 90% of CD8 T cells will succumb to

apoptosis. 3) The surviving cells with memory properties are then able to prosiest through cytokine-mediated homeostatic proliferation. 4) If the antigen is re-encountered, memory T cells can regain effector function and proliferate extensively to form a robust secondary response.

1.2.7 CD8 T cell differentiation

CD8 T cells can be classified into a range of subsets that have identifiable phenotypes (**Table 1.1**) associated with particular functions and anatomical localisation. Upon leaving the thymus, naïve CD8 T cells (T_n) migrate to secondary lymphoid tissues, such as the spleen and lymph nodes, with the phenotype of $CCR7^+$, $CD62L^+$, $CD27^+$, $CD28^+$, with $CD44^-$ in mice and $CD45RA^+$ in humans. Encountering a cognate antigen alongside appropriate stimulation can result in the activation of a naïve T cell, initiating various changes in gene expression. Within a CD8 T cell immune response, activated cells will rapidly proliferate to generate a large number of cells with effector phenotypes, which are then seen to contract following antigen clearance leaving a much lower number of cells with a memory profile that offer long-term protection from re-exposure (**Figure 1.4**). Following T cell activation there can be considerable heterogeneity between different CD8 T cell subsets capacity to migrate to peripheral sites, meditate effective killing, survive, and proliferate. Classically, short-lived effector T cells (T_{eff}: $CD127^-$ $KLRG1^+$) have been shown to have a far greater ability to kill target cells compared to and memory precursor effector T cells (MPECs: $CD127^+$ $KLRG1^-$) that possess increased proliferative potential, although recent studies have also highlighted the use the $CD43^-$ $CXCR3^+$ phenotype to delineate CD8 T cells with greater ability proliferate upon restimulation compared to the $CD43^-$ $CXCR3^-$ phenotype that infers improved cytotoxic abilities [42-45]. The majority of cells possessing a T_{eff} phenotype are coincided to be terminally differentiated, as they have a very poor ability to persist. Indeed, only 5-10% of CD8 T cells survive the contraction phase that follows the antigen clearance, forming the memory population. These memory cells provide protection following antigen re-exposure, due to their increased frequency and more rapid response upon stimulation compared to naive cells. Various subsets of memory cells persist, which include resident memory (T_{rm}), effector memory (T_{em}), central memory (T_{cm}), and stem cell memory (T_{scm}) subsets. The T_{scm} subset consists of antigen-experienced cells that have retained a T_n anatomical distribution and phenotypic profile, $CCR7^+$, $CD27^+$, and $CD28^+$, with increased CD95 expression. The T_{cm} subset is shown to upregulate marker of activation, $CD44^+$ in mice and $CD45RO^+$ in humans, with the ability to express low levels of cytokines and retaining the expression of lymphoid homing markers $CD62L^+$ and $CCR7^+$ [46]. As such, T_{cm} cells are enriched in the lymph nodes relative to T_{em} subset that downregulates lymphoid homing markers in favour of chemokine receptors that facilitate trafficking to peripheral sites of inflammation. While patrolling peripheral tissue, T_{em} subset exhibit effector function with the

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ability to express high levels of IFNγ and granzyme B. The T_{em} subset is also characterised by shorter telomeres in humans and lower proliferation potential upon restimulation compared to T_{cm} [47]. Although T_{em} cells have the capacity to monitor peripheral sites, the tissue-resident T_{rm} subset also provides regional immunity, particularly for mucosal and epithelial tissue [48]. There is substantial heterogeneity within this subset, but $CD103^+$ (αE integrin) and $CD69^+$ can be used to phenotypically characterise T_{rm} cells [48].

		T_n	T_{scm}	T_{cm}	T_{em}	T_{eff}
Phenotype	KLRG1	-	-	+/-	++	+++
	CD127	+	++	+	+/-	-
	CD62L	+++	+++	++	-	-
	CD27	+++	+++	++	+/-	-
	CD28	++	+++	+++	+/-	-
Function	IFNγ	-	+	++	+++	+++
	IL-2	-	++	+++	+/-	+/-
	Cytotoxicity	-	+/-	+	+++	+++
	Telomere length	+++	+++	++	+	-
	Self-renewal	+	+++	++	+	-
Transcription factors	Eomes	+	++	++	++	++
	T-bet	-	+	++	+++	+++
	ID3	+++	+++	++	+	-
	ID2	+	+	++	+++	+++
	TCF7	+++	+++	++	+	-
	Blimp-1	-	+	+	+++	+++
	LEF-1	+++	++	+	-	-
	TCF8	-	+	++	+++	+++

Table 1.1: Markers of CD8 T cell differentiation. Phenotypic markers, functional markers and transcription factors are shown with their expression levels for T_n, T_{scm}, T_{cm}, T_{em}, and T_{eff} subsets. Information has been adapted from a recent review by Gattinoni et al. [49]

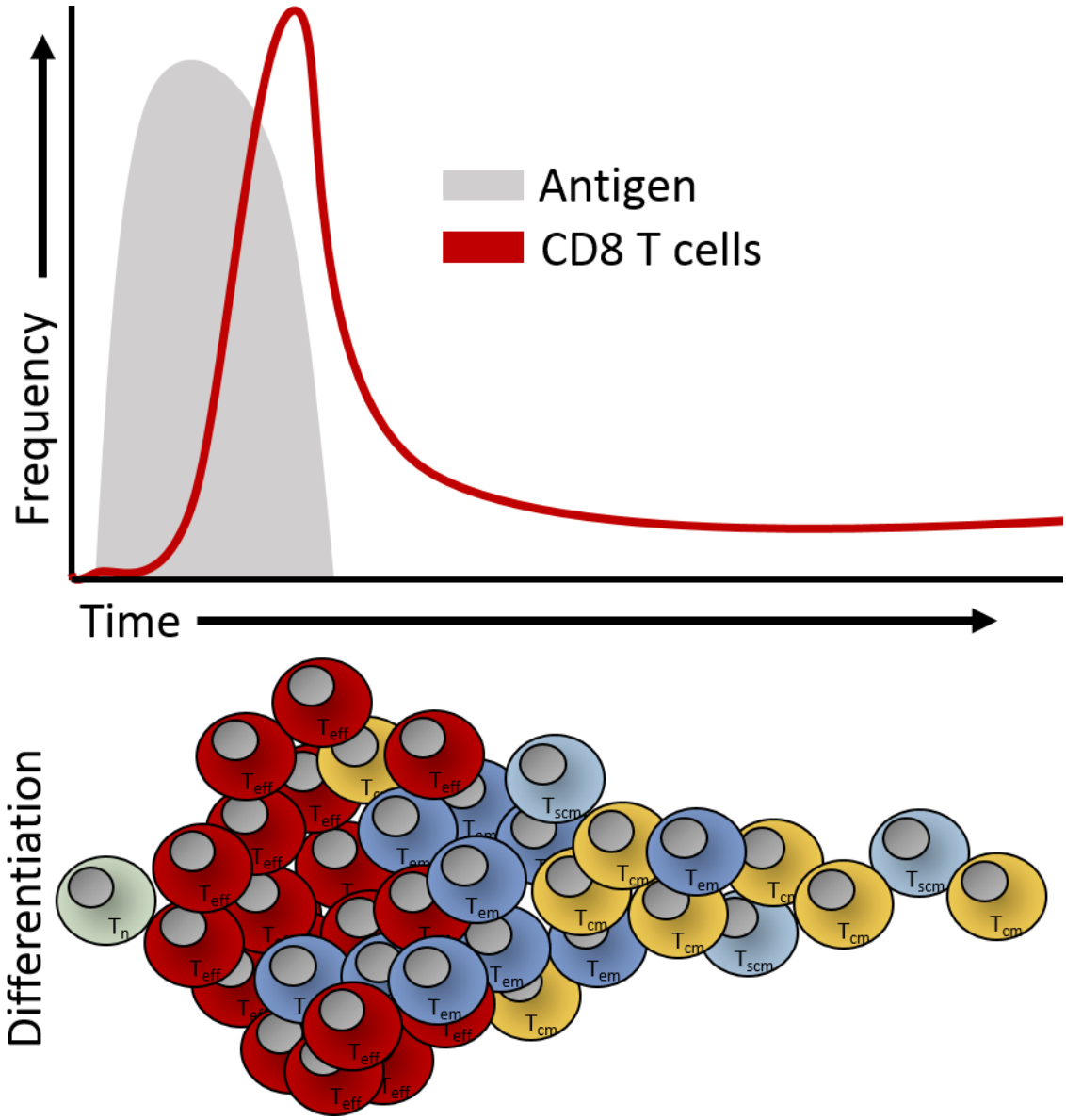

Figure 1.4: CD8 T cell differentiation within an immune response. Following adequate activation of naïve CD8 T cell, rapid proliferation results in the formation of a large population of effector cells. As antigen is cleared, these cells will no longer persist resulting in memory phenotypes becoming predominate within the population.

Various developmental mechanisms have been proposed to explain how heterogeneous populations of effector and memory T cells can form during an immune response. Firstly, it is worth noting that the separate precursor model, where cells are predestined during thymic development to differentiate into a given subset, has been disproved by studies utilising the adoptive transfer of single naive CD8 T cells, which give rise to both T_{em} and T_{cm} populations (**Figure 1.5A**) [50, 51]. The signal-strength model states that it is the overall strength of the initial priming from signals 1, 2, and 3 that is the foremost contributing factor in the differentiation of

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the CD8 T cells (**Figure 1.5B**) [52]. This allows for the formation of a heterogeneous population but is not particularly adaptable to changes in the disease status post T cell priming. The stepwise and flexible mechanism of the decreasing-potential model postulates that incremental advances are made towards terminal differentiation with repeated stimulation for signals 1, 2, and 3 (**Figure 1.5C**). This mechanism also allows for a spectrum of differentiation to occur, scaling effector response to the intensity of the disease as it progresses. This theory is supported by evidence of T cells acquiring high levels of effector function, reducing their capacity to form long-lived memory [53]. As such, reducing the duration or the intensity of an infection has been shown to accelerate CD8 T cell memory formation, and similarly, T cells that are activated during the later stages of an infection with lower inflammation levels exhibit preferential differentiation into a T_{cm} phenotype [53-55]. Finally, the asymmetric fate model, which is not mutually exclusive with the other models, could provide a mechanism by which dividing cells receive different levels of stimulation (**Figure 1.5D**). This theory states that the daughter cell proximal to the immune synapse receives stronger stimulation allowing for more effector differentiation compared to the distal daughter that will receive less stimulation and therefore has a greater potential to form memory [56]. Evidence for this model also comes from the asymmetric inheritance of other proteins that can govern differentiation, such as mTORC1 [47].

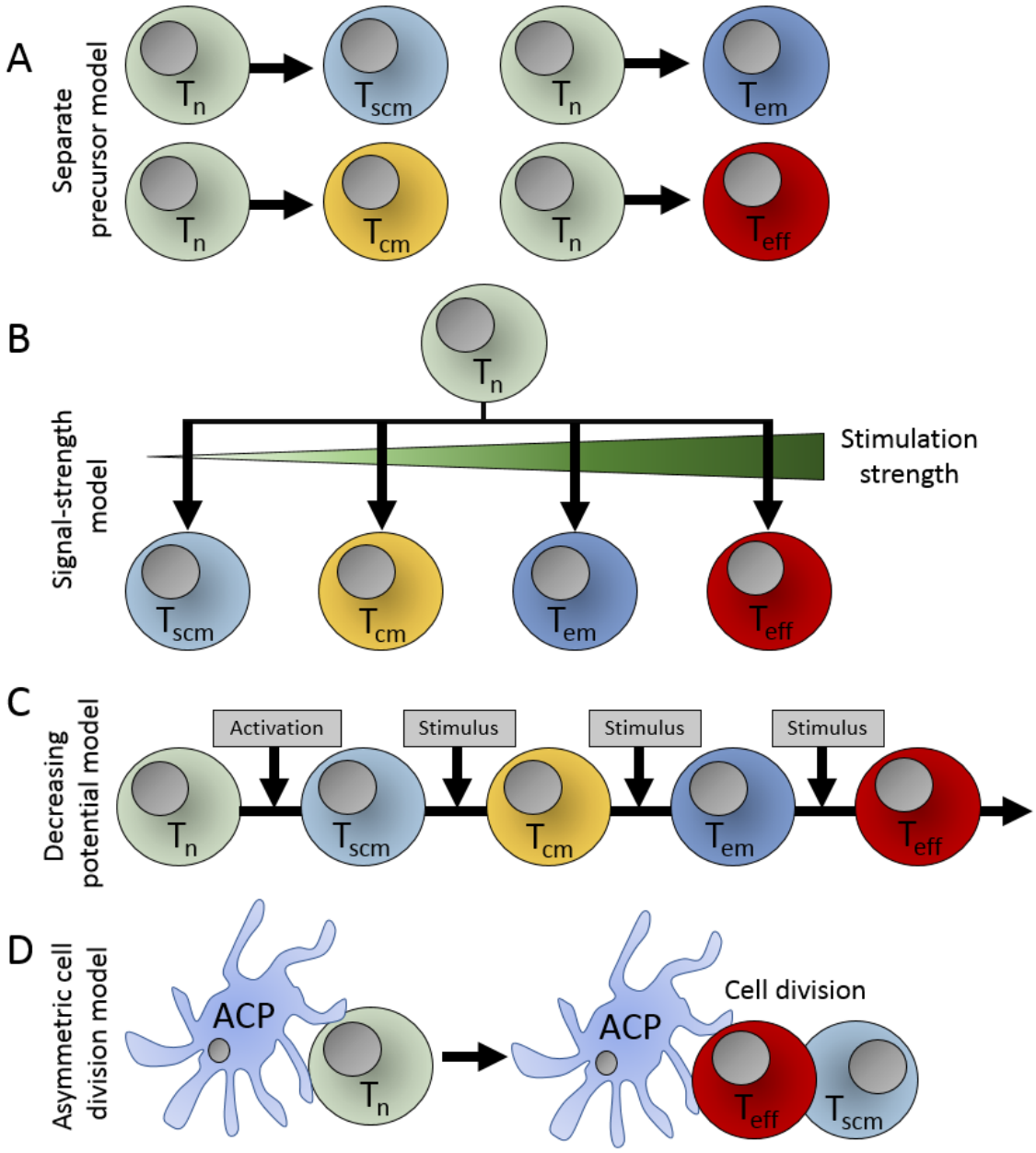

Figure 1.5: CD8 T cell differentiation models. Several models have been described for the formation of a heterogeneous T cell population following activation. A, The separate precursor model, where each naïve cells is predestined to differentiate into a given subset. B, The signal-strength model where the level of initial stimulation dedicates the differentiation status. C, The decreasing potential model, where T cells progress in a stepwise becoming more differentiated as they encounter further stimulation. D, The asymmetric cell division model, in which the daughter cell that inherits the immune synapse will gain greater stimulation than the distal daughter cells and therefore undergoes greater levels of differentiation.

1.2.8 Transcription factors guiding CD8 T cell differentiation

A plethora of extracellular signals arising from the TCR, costimulation, and cytokines utilise intracellular signalling pathways coordination of CD8 T cell differentiation. As such, the

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transduction of signals to transcription factors regulates CD8 T cell function and fate through the control metabolism, survival, effector function, and migration. For instance, the FOXO family of transcription factors coordinate aspects of the cell cycle, apoptosis, and lymphoid homing in T cells [57]. Additionally, several pairs of regulatory transcription factors have been described as forming an axis that distinguishes effector verses memory potential. These include B lymphocyte-induced maturation protein (Blimp)-1 and BCL6, inhibitor of DNA binding (ID)2 and ID3, and T-bet and Eomes. Understanding the role of these transcription factors in the context of a complex signalling network is important for understanding the how CD8 T cell differentiation is orchestrated and ways in which it can be manipulated.

1.2.8.1 T-bet and Eomes

The T-box transcription factors T-bet and Eomes are critical factors in regulating CD8 T cell effector function and the formation of memory. Upon activation, T-bet and Eomes are both upregulated and have an overlapping function for the expression of certain effector molecules, such as IFNγ and granzyme B, as well as chemokine receptors CXCR3 and CXCR4 [45, 51]. However, the relative expression of these factors at later stages of the immune response can dramatically influence CD8 T cell fate. T-bet expression is induced by TCR stimulation and inflammatory cytokines, such as IL-12 [53, 54]. The ratio of T-bet to Eomes is an important factor in CD8 T cell differentiation, as illustrated by several murine knockout models. T-bet deficiency impairs the ability of CD8 T cells to form effector cells, while T-bet overexpression results in an increased accumulation of effector subsets that have a reduced ability to persist into long-term memory. Conversely, CD8 T cells that are deficient in Eomes can undergo normal primary expansion following an acute infection but have reduced ability to form T_{cm}, impaired long-term survival, and reduced re-expansion capabilities [51]. While clearly impotent for memory formation, Eomes expression can promote aspects of effector function such as IFNγ expression and has been shown to compensate for the lack of T-bet, by facilitating anti-tumour immunity within a murine model [58]. The role of Eomes controlling T cells function has also been explored by overexpressing Eomes, resulting in the improved the accumulation of T cells in response to peptide stimulation *in vitro* and *in vivo* [59]. Taken together, the evidence suggests that T cell stimulation drives T-bet:Eomes ratios, which are of great importance for controlling the function and differentiation of these cells.

1.2.8.2 ID2 and ID3

ID2 and ID3 regulate the differentiation of CD8 T cells through the inhibition of E-protein transcription factors. Despite this similarity, the coordination of CD8 T cell survival and differentiation by ID2 and ID3 is strikingly different. ID2 controls apoptotic factors, such downregulating BIM and promoting BCL2 expression, while ID3 supports genome stability and DNA replication, through the enhances expression factors such as FOXM1 and NEK2 [60]. As such, ID2 promotes the survival of effector CD8 T cells in the primary response [61], while ID3 is important for supporting the survival of memory cells [62]. Although ID2 promotes accumulation of T cells during the initial stages of infection it also induces CD8 T cells to adopt effector phenotype ($KLRG1^+$ $CD127^-$) accounting for higher levels of terminal differentiation [60]. Opposingly, ID3 promotes T cell memory formation ($KLRG1^-$ $CD127^+$), which confers reduced levels of apoptosis during the contraction phase [60]. The ratio of ID2 to ID3 expression can therefore be used as a read-out of CD8 T cell differentiation even during early stages of clonal expansion.

1.2.8.3 Blimp-1 and BCL6

Blimp-1 and BCL6 have also been paired as antagonist transcription factors that govern the fate of CD8 T cells [63]. The Blimp-1 expression is high within the effector stages of immune responses but is downregulated as the memory population forms alongside the progressive accumulation BCL-6 [64]. Functionally, the loss of Blimp-1 in CD8 T cells hinders their ability to clear influenza virus, reduces their cytotoxicity, and limits their migration to the lungs [65]. Additionally, the absence of Blimp-1 results in an increase in T_{cm} accumulation following an LCMV infection [66]. Opposing the activity of Blimp-1, BCL-6 is a critical determinant of T_{cm} maturation [67]. Indeed, BCL-6 overexpression results in enhanced T_{cm} accumulation [68]. In Th1 cells, BCL-6 has been shown to compete with T-bet to block the DNA binding domains for certain genes (NOR1, SMRT, and BCOR) [69, 70]. There is therefore complexity beyond simple pairs of transcription factors, with intricate overlapping regulatory functions of many factors governing the transcriptome.

1.2.9 FOXO proteins in T cell differentiation and function

In CD8 T cells, activatory signalling pathways from the TCR, as well as various cytokine receptors and costimulatory receptors, converge on the activation of PI3K/Akt pathway, which then regulates the localisation and transcriptional activity of FOXO proteins (**Figure 1.6**) [71]. The pathway cascades as follows: Activated PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 allows the co-recruitment of Akt and phosphoinositide-dependent protein kinase 1 (PDK1) to the plasma membrane, through their pleckstrin homology (PH) domain. PDK1-dependent phosphorylation of

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Thr308 is crucial for Akt activity. For maximal activation, however, Akt is also phosphorylated by mTORC2 at Ser473. Activated Akt can phosphorylate three sites of FOXO1 (murine sites: Thr24, Ser253 and Ser316) and FOXO3 (murine sites: Thr32, Ser253 and Ser315) [72]. Phosphorylated FOXOs bind to a 14-3-3 scaffolding protein, which induces their nuclear export and results in subsequent degradation by ubiquitination [73].

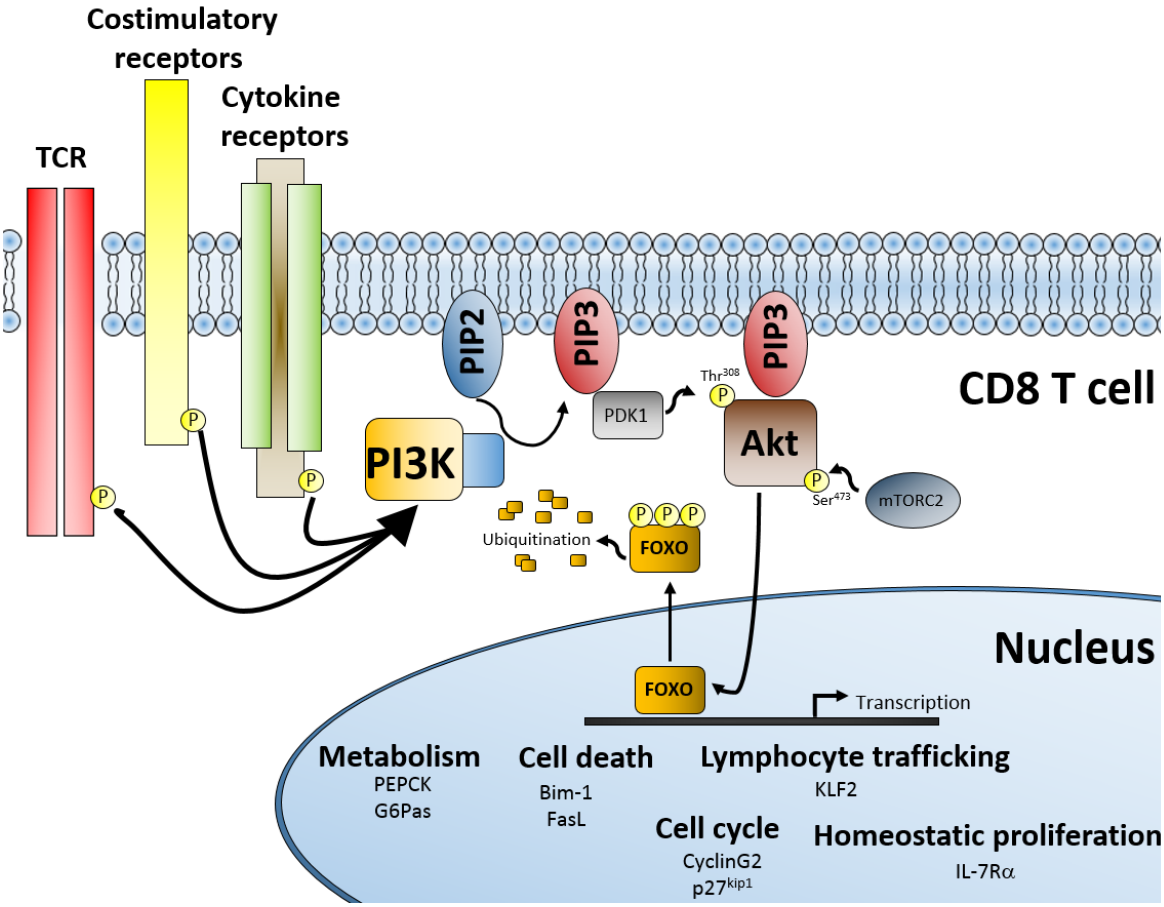

Figure 1.6: FOXO signalling pathway. CD8 T cell stimulation form signal 1 (TCR), 2 (co-stimulation receptors), and 3 (cytokine receptors) converge on PI3K activation, which mediates the phosphorylation of PIP2 to generate PIP3. PIP3 allows for the co-localisation of PDK1 and Akt at the cells intracellular membrane. Activated Akt, phosphorylated by PDK1 and mTORC2, downregulates FOXO activity by phosphorylating FOXOs at three sites, which induces its nuclear export and results in its degradation by ubiquitination. FOXOs proteins regulate many families of genes including those involved in ROS detoxification, metabolism, cell death, cell cycle inhibition, lymphocyte trafficking, and homeostatic proliferation.

1.2.9.1 FOXO transcription factors

The FOXO proteins are a family of transcription factors that are expressed in many cell types and persist in a hypophosphorylated state in the nucleus of quiescent cells, where they regulate the

expression of numerous genes involved in proliferation, metabolism, cell growth, differentiation, and longevity [74]. This study focused on FOXO1 and FOXO3, which have been shown to control aspects of CD8 T cells differentiation and survival [57, 75, 76].

1.2.9.2 FOXO1

FOXO1 regulates a wide variety of genes involved in metabolism (Phosphoenolpyruvate carboxykinase (PEPCK) and Glucose 6-phosphatase (G6Pase)), cell death (Bcl-2, BIM, and FasL), differentiation (BTG-1, p21, and Eomes), ROS detoxification (catalase), and cell-cycle inhibition (Cyclin G2 and $p27^{Kip1}$) [57, 73]. Recently, *in vitro* and *in vivo* studies have also implicated FOXO1 as an important factor in determining naïve CD8 T cell homeostasis and trafficking by regulating IL-7Rα and Kruppel-like factor 2 (KLF2) respectively [76-79]. The loss of FOXO1 causes a reduction in IL-7Rα expression, which results in lower levels of the antiapoptotic factor Bcl-2 due to decreased IL-7 signalling [78]. The loss of FOXO1 expression also reduces the levels of KLF2, which in turn reduces the expression of the lymphoid homing molecules CD62L and CCR7 [77, 78, 80]. Although the global loss of FOXO1 is lethal in mice, due to vascular depletion, several studies have utilised mouse models with a conditional deletion of FOXO1 in T cells ($CD4^{cre}$ $FOXO1^{fl/fl}$ knockout) or activated CD8 T cells ($GzmB^{cre}$ $FOXO1^{fl/fl}$ knockout). $FOXO1^{-/-}$ CD8 T cells expand normally following an LM infection, but the cells that persist post antigen clearance fail to respond to secondary challenges [81]. Similarly, in an LCMV infection setting the FOXO1 deficient CD8 T cells initially undergo normal differentiation but then continue to exhibit an effector phenotype (granzyme B^+, $CD69^+$, and $TCF7^-$) post antigen clearance, failing to form functional memory cells [79, 82]. In contrast to WT cells, FOXO1 deficient CD8 T cells have reduced PD-1 expression and fail to persist in a chronic LCMV model [83]. Therefore, FOXO1 can function to desensitise CTLs to antigens and supports survival during chronic infection. A study by Shrikant's group has also demonstrated that FOXO1 overexpression in CD8 T cells causes an upregulation of Eomes while repressing T-bet, granzyme B, and IFNγ expression following an *in vitro* activation of cells alongside IL-12 [55]. Taken together, FOXO1 promotes transcriptional programs that favour the differentiation of memory cells at the cost of effector function. Therefore, there is potential to manipulate FOXO1 in order to guide CD8 T cells into a memory profile prior to ACT.

1.2.9.3 FOXO3a

The FOXO3a transcription factor regulates the expression of several genes involved in cell cycle arrest (p21, p27 and BCL6), cell death (BIM, PUMA, PTEN and FasL), stress resistance (Catalase and SOD2), and cell metabolism (UCP2) [84-87]. Several groups have characterised FOXO3

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deficient CD8 T cells using infection models. The LCMV models have illustrated that a lack of FOXO3 increases the expansion of CD8 T cells during the primary response, due to reduced apoptosis [88-90]. In one report the enhanced survival was attributed to an extrinsic mechanism, as FOXO3$^{-/-}$ CD8 T cells did not possess improved survival when adoptively transferred to WT C57BL/6 mice, and it was argued that the improved survival could have resulted from an increase in IL-6 production by FOXO3$^{-/-}$ DCs [89]. However, another group reported a CD8 T cell-intrinsic mechanism for enhanced survival during the primary expansion of cells linked to reduced BIM expression in FOXO3 deficient T cells [88]. Recently, it has been shown that FOXO3 deficient CD8 T cells undergo greater primary expansion and persist at higher levels post contraction compared to WT controls, in both infectious (vaccinia virus) and non-infectious (non-replication cellular vaccine) models [91]. However, the enhanced frequency of cells did not result in an improved secondary expansion following a vaccine rechallenge [91]. This suggests that the cells possessing FOXO3a could form more functional memory cells and that this compensated for the lower frequency of cells observed. Finally, the primary expansion was unchanged for FOXO3a deficient CD8 T cells in an LM infection model, yet these cells persisted at higher levels following the contraction attributed to reduced BIM and PUMA expression [92]. Taken together, these data indicate that FOXO3a functions as a negative regulator of CD8 T cell survival yet its function is context dependent. As such, FOXO3a requires further investigation to fully elucidate its role in T cell survival versus functional differentiation especially outside of the context of infectious models where data are lacking.

1.2.10 Protein tyrosine phosphatases regulation of T cell stimulation

PTPs directly oppose the action of kinases, reversing tyrosine phosphorylation through the hydrolysis of a phosphoric acid monoester on their target substrate. As phosphorylation serves as a fundamental mechanism for regulating protein function, there are a large number of highly specific kinases and opposing phosphatases [93]. Although tyrosine phosphorylation accounts for less than 2% of the phosphoproteome, PTPs are critical regulators of fundamental cell functions and physiology [94]. While some PTPs act to promote T cell activation, others inhibit this process. The PTP family includes membrane-bound PTPs such as CD45, as well as PTP non-receptors (PTPNs), which mainly reside within the cytosol. Importantly, TCR activation as well as the triggering of many co-stimulatory receptors and cytokine receptors, initiate intracellular signalling through the phosphorylation of tyrosine residues within ITAM domain and are therefore subject to PTP regulation [95].

As regulators of ITAMs, PTPNs serve as key components in metabolism, effector function, and differentiation following CD8 T cell activation. For example, the phosphorylation of ITAMs on Lck and Fyn are critical steps for the initiation of TCR signalling resulting in the recruitment and activation of Zap70, which transduce signals to adaptor proteins that regulate T cell function (see **Chapter 1.2.3** for full details on TCR signalling). The regulation of ITAMs is complicated by the fact that various phosphotyrosine motifs can exist for each molecule, which may be targeted by one or more phosphatase. For instance, within the TCR signalling pathway PTPN2 and PTPN22 dephosphorylate the same activatory residues on Fyn and Lck, while PTPN22 independently inhibits ZAP70 (**Figure 1.7**) [96, 97]. Additionally, several PTPNs regulate cytokine signalling pathways. Cytokine receptors do not have intrinsic PTK activity but utilise activatory tyrosine residues on Janus kinases (JAKs), which signal to the substrates signal transducer and activator of transcription (STAT) family following cytokine engagement [98]. By controlling the phosphorylation of JAK and STAT proteins, several PTPNs regulate signalling following IL-2, IL-7, IL-12, and IL-15 receptor engagement, thereby controlling T cell survival and proliferation [99]. The importance of several phosphatases, including PTPN2, PTPN6, and PTPN22, have been highlighted as their dysregulation is linked to T cell hyperresponsiveness and the development of autoimmune responses [100, 101].

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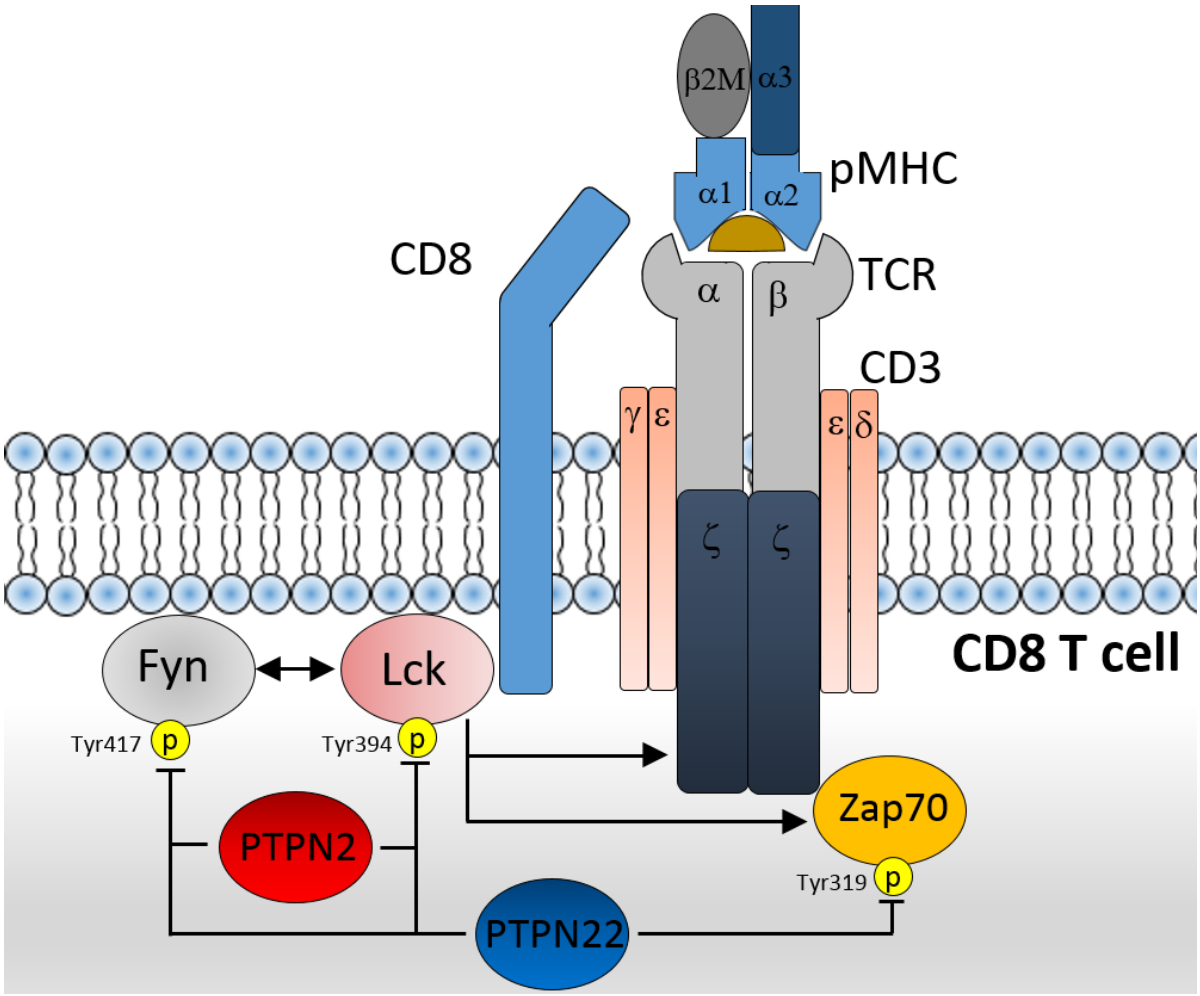

Figure 1.7: PTPN2 and PTPN22 suppression of TCR signalling. By inhibiting activatory residues on Fyn and Lck, PTPN2 and PTPN22 limit the phosphorylation of ITAMs on CD3 chains and reduces the activation of ZAP70. In addition to this, PTPN22 also directly dephosphorylates Zap70.

Due to the thymic selection process, CD8 T cells have a degree of reactivity towards self-antigen presented by MHC class I molecules. Lymphocyte activation is therefore a tightly regulated process, ensuring a balance between proving sufficient sensitivity towards pathogens and avoiding autoimmune responses while. Peripheral tolerance is, in part, dependent on the signalling cascades following TCR engagement discriminating between the frequent low-affinity interactions with self-peptides and infrequent interactions with higher affinity pathogen-derived antigens [100, 102]. In theory, many tumour-derived peptides could bind a TCR to initiate an effector response [103-105]. However, unlike antigens derived from foreign pathogens with high affinities for TCRs, reaching KDs around 1µM, tumour-associated peptides are or are closely related to self-peptides and typically display much lower KDs of 10-100µM [106]. Weak interactions are mainly due to the fact that T cells expressing TCRs that would have strong self-antigen interactions are deleted or suppressed through central and peripheral tolerance

mechanisms. The sub-optimal TCR stimulation combined with other tolerising factors in the tumour microenvironment can therefore dramatically limit the generation of effective anti-tumour T cell responses (See **Chapter 1.3.7** for full details) [107]. The selection of high-affinity TCRs, obtained through screening or mutagenesis, can be utilised to improve the efficacy of ACT [108-110]. For example, in the development of the MAGE-A3 TCR, it was shown that the A118T substitution in the CDR3 region of the α-chain was able to outperform its WT counterpart in functional assays [111]. However, the enhancement of T cell sensitivity is accompanied by an increased risk of off-tumour reactivity. Indeed, TCRs that bind pMHC at very high affinity (KD < 1nM) have been shown to lose specificity [106], and several recent incidences have highlighted that use of T cells transduced with high-affinity TCRs and CARs can result in fatal off-tumour toxicity following ACT [112]**.**

Elucidating molecular mechanisms to enhance the intracellular signalling in response to TCR stimulation may offer opportunities to overcome low-affinity peptide interaction or immunosuppressive factors that raise T cell activation thresholds. An advantage of this approach would be the simplicity of applying a universal augmentation to a heterogeneous population of autologous TILs or neoantigen reactive T cells without having to develop a specific high-affinity TCR for each cancer. Promising progress for this approach has been made by Wucherpfennigs and colleagues, who used a sophisticated shRNA screening process that identified Ppp2r2d as an intrinsic immunosuppressive factor that limits CD8 T cell accumulation at the tumour site [113]. This study went on to show that the knockdown of Ppp2r2d, a regulatory subunit of the phosphatase PP2A, enhanced the proliferation, cytokine production, and anti-tumour capabilities of the CD8 T cells in a murine ACT model [113]. Exploring whether the inhibition of other phosphatases can enhance T cell sensitivity to augment ACT therapy is therefore a worthwhile area of research to investigate further.

Genetic and pharmacological modulation of several phosphatases have already been shown to enhance TCR sensitivity and improve the functionality of T cells. Recent work on PTPN6 (Shp-1), which dephosphorylates activatory residues of Lck and Zap70, has demonstrated that the lymphocytes of PTPN6$^{-/-}$ mice undergo greater levels of TCR mediated proliferation within an acute LCMV infection compared to WT T cells [114]. Additional, PTPN6$^{-/-}$ T cells have an improved ability to media effective anti-tumour immunity in ACT models alone or in the context of providing exogenous IL-2 [115]. Furthermore, the CD8 T cells transduced with siRNA targeting PTPN6 (achieving a 66% knockdown), enhanced the *in vivo* proliferation and the killing ability of cells [115]. Within this report, I assessed the functional consequences of inhibiting the

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phosphatases PTPN2, PTPN7, and PTPN22 in CD8 T cells in the context of ACT therapy against cancer.

1.2.10.1 PTPN2

PTPN2 (also known as T cell-PTP, despite being expressed in many cell types) is a cytosolic phosphatase that acts as an important negative regulator for both TCR [116] and JAK/STAT signalling [117, 118]. Single nucleotide polymorphisms (SNPs) that reduce PTPN2 activity are associated with various autoimmune diseases. For instance, the loss of function SNP rs1893217(C) carries approximately a 1.3-fold increase in the occurrence of diabetes, rheumatoid arthritis, and Crohn's disease [119]. The expression of PTPN2 is elevated in naive T cells leaving the thymus, which is thought to restrict proliferation and limit their response to self-antigens [96]. Interestingly, in comparison to naïve cells, memory CD8 T cells express higher levels of PTPN2, which may partly account for the lower levels of Zap70 phosphorylation observed in this population following TCR stimulation [120]. Recent efforts have been made to characterise target proteins and elucidating function of PTPN2 in TCR and cytokine signalling through the use of PTPN2 substrate-trap mutants and PTPN2-deficient mouse models.

Several cytokines signalling pathways that govern the survival, proliferation, and function of T cells are regulated by PTPN2. For example, PTPN2 inhibits activatory residues on JAK1 and JAK3. As such, enhanced STAT5 phosphorylation is observed in PTPN2$^{-/-}$ T cells following IL-2 stimulation [118]. However, as PTPN2$^{-/-}$ T cells upregulate CD25 expression to higher levels following TCR stimulation, it is difficult to assess from these experiments whether the difference in STAT5 phosphorylation can be attributed to direct increases signalling intensity from the lack of PTPN2 suppressing STAT5 or simply greater levels of CD25 interaction [116]. Human studies support the claim that PTPN2 deficiencies enhance JAK1 signalling, linked to increased proliferation and disease progression of T cell acute lymphoblastic leukaemia (T-ALL) [121]. Additionally, PTPN2 suppresses inflammation by limiting STAT1 and STAT3 phosphorylation following IFNγ stimulation of T cells [117]. Interestingly, in a murine ACT therapy model, the overexpression of miR-155 in CD8 T cells improved anti-tumour immunity, which was attributed partly to the lower levels of PTPN2 and subsequent increase in STAT5 phosphorylation following cytokine stimulation [122]. Enhanced sensitivity to cytokine stimulation is particularly beneficial in the context of ACT protocols when lymphopenia is induced to facilitate increased cytokine availability *in vivo* [122]. In one study, naïve PTPN2 deficient T cells underwent greater proliferation and STAT5 phosphorylation in response to IL-7 stimulation [123]. In contracts, another group has shown that

STAT5 phosphorylation is unchanged following IL-7 or IL-15 in PTPN2$^{-/-}$ T cells [96]. Here antigen-experienced PTPN2$^{-/-}$ T cells did not undergo enhanced LIP, and the phenomenon in naïve cells was attributed to PTPN2s ability to regulate TCR signalling following weak self-peptide interaction [96].

PTPN2 dephosphorylates activatory residues of Lck (Tyr394) and Fyn (Tyr417), thereby suppressing fundamental components of TCR signalling [124]. The importance of PTPN2 regulating the responsiveness of immune cells is highlighted by the global loss of PTPN2 in mice resulting in severe systemic inflammation and autoimmunity symptoms within 1-2 weeks of birth, leading to fatal anaemia within 5 weeks [125]. This condition is attributed to the hyper-reactivate immune cells across several lineages, including myeloid cells, B cells, and T cells [126, 127]. The impact of T cell-specific PTPN2 deficiency is less severe, as mice are healthy until at least 12 weeks, but by 40 weeks mice exhibit reduced body weight and approximately 40% mice succumb to lethal autoimmune responses [116]. Intrinsically, PTPN2$^{-/-}$ T cells transition away from a naive phenotype towards effector (KLRG1^{+} CD127^{-}) and memory (CD62L^{+} CD44^{+}) profiles at a faster rate than in WT mice [116]. These PTPN2 deficient T cells also possess enhanced sensitivity towards TCR stimulation. While the levels of Lck (Tyr394) and Fyn (Try417) phosphorylation was unaltered within resting naïve PTPN2$^{-/-}$ CD8 T cells, they were enhanced, alongside increased activation of downstream factors such as ERK1/2, following TCR stimulation [116]. The increased activation status of PTPN2$^{-/-}$ CD8 T cells was illustrated phenotypically through the increased upregulation of activation markers, CD44, CD69, and CD25 [116]. Functionally, the increased activation of PTPN2$^{-/-}$ T cells, resulted in increased proliferation following αCD3 stimulation with or without αCD28 costimulation [116]. Interestingly the largest relative increase in activation profiles and proliferation of these cells compared to their WT counterparts was achieved using the lower levels of stimulation.

Work conducted by Tiganis and colleagues have gone on to claim that PTPN2 plays a critical role as gatekeeper for weak TCR interactions for both naïve and antigen-experienced CD8 T cells. To assess the role of PTPN2 in the response of TCR signalling towards different affinity antigens, the PTPN2 deficiency was crossed onto an OT-I background [128]. Firstly, PTPN2 deficiency in naïve OT-I CD8 T cells was shown to enhance proliferative capabilities towards OVA or SIIN peptide vaccination following adoptive transfer into WT mice [116]. When lower affinity peptides, SIIT and SIIG, were used during *in vivo* vaccination experiments the relative enhancement in proliferation afforded by PTPN2 deficiency was increased [116]. This finding was also supported by *in vitro* assays where the use of a low-affinity peptide gave the greatest differences in proliferation

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between WT and PTPN2$^{-/-}$ naïve OT-I [116]. Here, the influence of PTPN2 suppressing responses to weak TCR stimulus was retained by memory cells, as PTPN2$^{-/-}$ T_{cm} had enhanced proliferation following low levels of αCD3 stimulation [96]. To further support the role of PTPN2 in maintaining tolerance, it has been shown that PTPN2 deficiency is sufficient for OT-I cells to mediate diabetes following adoptive transfer into mice where the pancreatic β-cells express OVA [96].

Taken together, the current research indicates that PTPN2 can play an important role in regulating homeostasis, activation, and proliferation in CD8 T cells linked to its ability to suppress JAK/STAT signals and TCR signalling. Interestingly, a recent study validating a high throughput shRNA screening technique showed that the knockdown of PTPN2 could endow a 7.4-fold improvement in the accumulation of T cells at a tumour site following adoptive transfer, yet this finding was not investigated further [113, 123]. It is therefore of interest to investigate the effect of shRNA-mediated knockdown of PTPN2 in activated CD8 T cell differentiation and function to assess if this modification can provide a translatable benefit for ACT therapy against cancer.

1.2.10.2 PTPN7

The phosphatase PTPN7 is preferentially expressed in hematopoietic lineages where it regulates several cellular processes, including pathways downstream of TCR signalling. For example, PTPN7 can directly bind to and inhibit substrates mitogen-activated protein kinase 1 (ERK2) and p38 [129]. Furthermore, the overexpression of PTPN7 in Jurkat cells results in reduces NFAT/AP-1 activity upon TCR stimulation [130]. PTPN7 is also upregulated following T cell activation and IL-2 stimulation, suggesting it may exert a negative feedback loop for ERK2 mediated signalling [131]. While T cells in PTPN7$^{-/-}$ mice have higher levels of NFAT activation upon αCD3 stimulation, lymphocyte differentiation following activation is largely unchanged, and thymocyte development is unaffected, suggesting that a mechanism of redundancy may be in place to compensate for the loss of PTPN7 in naïve cells [132]. Interestingly, PTPN7 plays a role in macrophages after LPS stimulation, where TNFα expression is inhibited by PTPN7 overexpression and enhanced following PTPN7 knockdown [133]. With limited studies investigating the functional role of PTPN7, further work is required to delineate whether it's manipulation may impact the sensitivity to TCR stimulation, differentiation, and function of activated CD8 T cells.

1.2.10.3 PTPN22

PTPN22 (also known as LYP in humans and PEP in mice) is a cytosolic phosphatase exclusively expressed in hematopoietic cells, where it regulates aspects of activation and homeostasis.

PTPN22's role in regulating T cell activation has been highlighted by the SNP C1858T, resulting in an R620W substitution, which is a major risk factor in autoimmune diseases such as rheumatoid/idiopathic arthritis, type 1 diabetes, systemic lupus erythematosus, vasculitis, and Grave's disease [134-136]. Indeed, these diseases have up to a 2.5-fold higher occurrence rate for carriers of the R620W mutation [137]. Despite this increased susceptibility to autoimmunity, epidemiological data show that the prevalence of this SNP has reached 15% in some European populations, indicating there may have been a selective historical bias favouring this trait [134]. For instance, it is possible that the increased sensitivity of the immune system could provide a protective benefit against certain infections thereby decreasing total mortality for R620W carriers.

The R620W mutation falls in the P1 region of PTPN22, which compromises its ability to dephosphorylate Lck and bind to CSK, a kinase that inhibits Lck activity [138]. The complete deletion of PTPN22s P1 domain shows the same impact as the R620W mutations, limiting CSK binding and reducing dephosphorylation activity against Lck activatory residue [97]. The loss of PTPN22 activity can facilitate increased levels of T cell activation following TCR engagement [139]. However, T cells acquired from human patients suffering an autoimmune disease have shown that the presence of R620W confers lower responsiveness to TCR stimulation in terms of calcium flux, proliferation, and IL-2 production [140]. This has led some researchers to claim that the R620W mutation could confer a gain of function effect in T cells to suppress TCR signalling. However, the reduced activation in these circumstances may also be indicative of exhausted T cell phenotypes. The stronger levels of tonic TCR signalling sustained by the T cells possessing R620W could promote a dysfunctional state mimicking the response seen in chronic infections [141]. Robust mouse models, using the murine R620W equivalent substitution (R619W), have confirmed this mutation causes a loss of PTPN22 function, as T cell differentiation and response to activation is similar to that observed in PTPN22 deficient mice. However, with conflicting evidence from mouse and human studies, further work on the molecular mechanisms by which the R620W mutation impacts PTPN22 function in an autoimmune setting is needed.

PTPN22 functions as a negative regulator of T cell stimulation by dephosphorylating the activatory tyrosine residue of Lck (Tyr394), Fyn (Tyr417), and other SFK substrates, such as Zap-70 (Tyr319) and E3 ubiquitin ligase c-Cbl[97, 116, 139, 142]. Similarly to PTPN2, PTPN22 is expressed at higher levels in memory CD8 T cells compared to naive cells and therefore may be another contributing factor in the lower levels of ZAP70 phosphorylation seen following their TCR stimulation [120]. Furthermore, similarly to PTPN2$^{-/-}$ mice, a greater number of PTPN22$^{-/-}$ T cells survive negative

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selection and show increased accumulation of T_{em} cells in peripheral tissues over time [139]. Unlike PTPN2 deficient mice however, the global loss of PTPN22 does not result in spontaneous autoimmunity, which has been attributed to a GITR dependent increase in accumulation for PTPN22$^{-/-}$ T_{regs} [143, 144]. Additionally, PTPN22 deficiency confers a faster STAT5 phosphorylation response to IL-2 stimulation in T_{reg} cell [145]. However, this may be due to the higher upregulation of CD25 seen following the activation of PTPN22$^{-/-}$ T cells. As such, both naïve and antigen-experienced PTPN22$^{-/-}$ T cells exhibit enhanced proliferation and expression of activation markers in response to stimulation with αCD3 and αCD28 compared to WT cells [139]. Interestingly, PTPN22 is particularly important for regulating TCR responses to low-affinity antigen interaction. Work conducted by Zamoyska's group has shown that peptide stimulation of naïve OT-I cells lacking PTPN22 results in enhanced proliferation and activation, with increased T-bet and c-Myc expression [100]. However, only the stimulation with the low-affinity peptides, SIIG or SIIT, enhanced the expression of IFNγ, IRF4, and LFA-1 [100]. As these factors are critical for T cell cytotoxicity, activation, and adhesion receptively, this implicates PTPN22 as a key negative regulator for low-affinity antigen interactions. In support of this, PTPN22$^{-/-}$ T cells proliferated to a greater extent and acquired greater effector function when transferred into lymphopenia conditions, including Rag$^{-/-}$, NSG, or irradiated C57BL/6 mice, attributed to increased responsiveness to weak self-antigen interactions [100].

Contrary to PTPN22's role in limiting responses to *in vivo* vaccination shown previously, within the context of inflammatory bacterial infections the presence of PTPN22 can benefit T cell function. Following acute LCMV infection, PTPN22 deficient T cells had reduced STAT1 phosphorylation and impaired accumulation within the primary expansion. Additionally, the PTPN22 deficient memory CD8 T cells generated following the LCMV infection have decreased cytokine expression upon restimulation [146]. This result contradicts previous observations and, therefore, highlights that phosphatases can perform different roles depending on the context of the immune response [147].

Collectively, research indicates that PTPN22 plays an important role in regulation TCR signalling, particularly suppressing weak interactions, influencing the ability of naïve, effector, and memory CD8 T cells to proliferate, produce cytokines, and express adhesion molecules. However, the role of PTPN22 appears to be context dependent, and it is not yet known how the knockdown of PTPN22 will affect the function of activated T cells in the context of ACT therapy against cancer.

1.2.11 CSK regulation of TCR signalling

Lck is known as a gatekeeper for TCR signalling due to its essential role in the phosphorylation of Zap70, which triggers the signalling cascade responsible for T cell activation [148]. The activity of Lck is therefore tightly regulated. The open conformation of Lck promotes auto-phosphorylation at its Tyr394 residue, which then facilitates the activation of ITAMs on CD3 zeta chains and Zap70 [149]. The downregulation of Lck activity can occur through the dephosphorylation of Tyr394 or by the phosphorylation of the Tyr505 site promoting the closed 'inactive' conformation, in which Tyr394 phosphorylation cannot occur. The Tyr505 site is regulated by the opposing actions of a PTP, CD45, and a PTK, CSK. By controlling Lck activity, both these factors have a crucial role in regulating TCR signalling. Despite the fact that CD45 can inhibit TCR signalling through the dephosphorylation of the Tyr394 site, the disruption of this protein results in Tyr505 hyper-phosphorylation rendering T cells non-responsive to TCR stimulation [150]. Likewise, the overexpression of CSK leads to reduced TCR signalling and T cell function, as shown by reduced IL-2 expression following stimulation [151]. Conversely, inhibiting CSK kinase activity results in increased Lck activity even in the absence of TCR activation [152]. As the activity of CSK is dependent on its cellular localisation, constitutively targeting CSK to the plasma membrane reduces T cell activation [152]. Localisation can be disrupted by inhibiting a membrane adaptor molecule that binds CSK, such as a phosphoprotein associated with membrane-associated glycosphingolipid-enriched microdomains (PAG), which results in enhanced proliferation of T cells in response to αCD3 stimulation [153]. It is interesting to note that PTPN22 binds to the SH3 domain CSK, with data indicating that these two factors may work synergistically to inhibit TCR signalling [97] (**Figure 1.8**). However, it is worth mentioning that other studies have concluded that PTPN22 activity is dependent on its disassociation from CSK [154]. With conflicting findings, further research is required to elucidate the role of the PTPN22-CSK complex.

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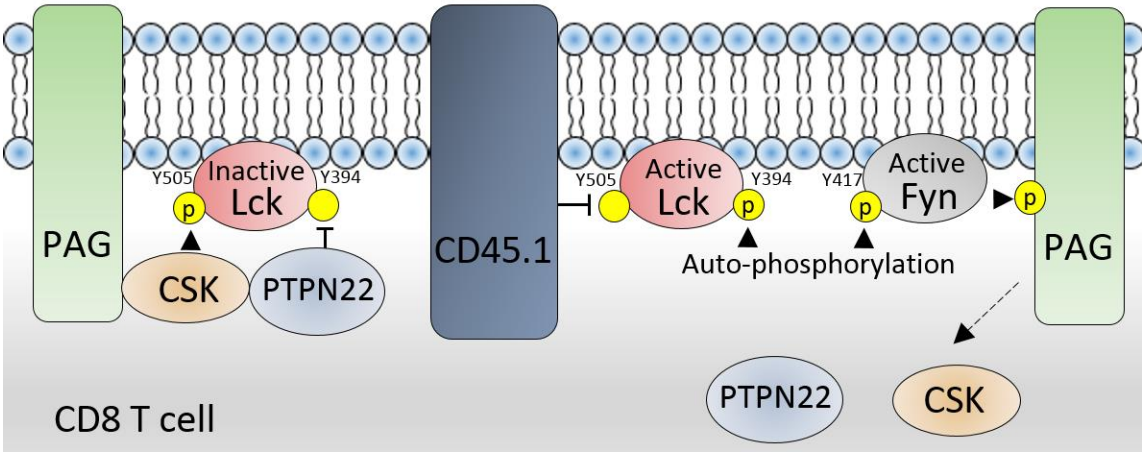

Figure 1.8: CSK inhibition of TCR Signalling. The localisation of CSK to membrane proteins, such as PAG, allows it to phosphorylate Lck at the Tyr505 site to promote an inactive conformation. Simultaneously, the SH3 domain of CSK allows for the binding to PTPN22, which deactivates Lck through the dephosphorylation of Tyr394. CD45 can dephosphorylate the Tyr505 site to promote an open conformation. In this state, TCR activation promotes auto-phosphorylation of Lck and Fyn, which facilitates the phosphorylation of PAG resulting in the release of CSK and its dissociation from the plasma membrane and PTPN22.

Sophisticated analysis of CSK inhibition in T cells has been conducted by Weiss and colleagues, who developed an analogue-sensitive form of CSK, CSK^{AS}, for which the kinase activity can be specifically inhibited by a small molecule. Utilising this technology, BAC transgenic $Csk^{-/-}$ mice that expressed CSK^{AS} were generated [155]. The inhibition of CSK^{AS} in T cells resulted in stronger and prolonged phosphorylation of ZAP-70, LAT, PLC-γ1, and ERK1/2 following αCD3 stimulation [156]. Functionally, the CSK^{AS} inhibition resulted in the improved proliferation of naïve T cells following low levels of anti-CD3 stimulation, although this effect was lost at higher levels of stimulation. It is interesting to note that the inhibition of CSK^{AS} did not dramatically reduce Tyr505 phosphorylation, but could mediate an up to a fourfold, dose-dependent, increase in Tyr394 phosphorylation. Utilising CSK^{AS} on an OT-I background, it was demonstrated that CSK inhibition gave a slight improvement to activation following strong stimulation, yet with weak stimuli, it was able to enhance T cell activation to a much greater extent, as measured by CD69 upregulation [156]. Caveats to this work include the fact that CSK is overexpressed within the control CSK^{AS} T cells likely portrays its inhibition as give a more striking than what would occur under philological conditions. However, this research does offer insight into the important role of CSK in limiting naïve T cells responses to low-affinity antigen. To further complicate matters, CSKs role in T cells activation may differ depending on the activation status of the T cell. Imaging studies have shown that CSK has a bipolar distribution in antigen-experienced cells as compared to naive cells when it is clustered at the immune synapse [157]. This localisation implies that CSK may be more

important on the TCR signalling of naïve T cells compared to antigen-experienced cells. While CSK can regulate TCR signalling, the extent to which its inhibition can be used to augment T cell function for ACT therapy against cancer is yet to be investigated.

1.2.12 Metabolism

The processes governing effector function and survival of CD8 T cells are intrinsically linked with metabolism. The metabolism of T_n cells consists of fatty acid oxidation and mitochondrial oxidative phosphorylation to generate ATP. As CD8 T cells become activated, a rapid switch in their metabolism to aerobic glycolysis and lipid synthesis occurs [158]. This switch enables activated cells to meet huge bioenergetic and biosynthetic demands that are required for extensive proliferation and production of cytotoxic molecules [159, 160]. The survival of T cells into memory is accompanied by a switch back to the catabolic status, primarily utilising oxidative phosphorylation. These metabolic transitions are regulated in part by the mTOR pathway, which is converged upon by many activatory signals, such as signals derived from TCR or IL-12R stimulation [55, 161]. The nutrient-sensing kinases mTORC1 and mTORC2 promote glycolysis, proliferation, and the development of effector CD8 T cells. As such, the inhibition of mTOR with low doses of rapamycin can promote the formation of memory T cells during inflammatory infections, such as LCMV [160, 162].

1.3 Tumour immunology and immunotherapy

Tumours are the result of genetically aberrant cells that have progressed in a multistep process towards achieving unrestricted proliferation [163]. When a neoplasm gains the ability to spread to other parts of the body, it is defined as cancerous. Cancers that reach this malignant stage have defining hallmarks that contribute to their continued growth; sustaining proliferative signalling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumour promoting inflammation, activating invasion and metastasis, inducing angiogenesis, resisting cell death, and deregulation of cellular metabolism [164]. Underlying these hallmarks is the genomic instability of the cancer cell, providing the mutations that can confer these selective advantages and thereby favour the accumulation of daughter cells. Mechanistically, this is achieved through random mutations in the DNA of a cell, such as insertions, deletions, and chromosome translocations, which enhance the expression of an oncogene or disrupts a tumour suppressor gene, leading to increased proliferation, survival, or mutation rates. Tumours are classified by the cell type from which they arise, which can be stratified into three main groups; carcinomas, sarcomas, and the lymphomas/leukaemias. The cancers of epithelial cells,

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carcinomas, constitute 90% of human cancers, the majority of which arise within the breast, prostate, lung, and colon [165]. Leukaemia and lymphomas are derived from immune cells and account for 8% of human cancers. Cancers from connective tissue, such as muscle and bone, are defined as sarcomas and have a comparatively low occurrence rate. Despite being defined within these groups, each individual cancer develops a different combination of mutations to achieve malignancy, resulting in heterogonous diseases in terms of presentation, development, and response to treatment. In the UK there are over 350,000 new cases of cancer diagnosed every year, and mortality due to cancer is responsible for over 29% of deaths (http://www.cancerresearchuk .org). As a major burden to health, economics, and society, it is of fundamental importance to understand optimal ways in which to treat cancers.

The body has many mechanisms for preventing the growth of cancerous tissues, including cell intrinsic defences such as regulating apoptosis in response to DNA damage or physiological stress, and extrinsic regulation by the immune system. The ability to evade the immune system has therefore been classified as a fundamental characteristic of cancer [164]. While the immune system has evolved in ways to recognise and eliminate cancer cells, the tumour microenvironment can utilise many mechanisms of evasion and tolerance to negate anti-tumour immunity. To overcome the suppressive tumour microenvironment novel immunotherapies are being developed to enhance activity and specificity of the immune system for the treatment of certain cancers [166].

1.3.1 Immunosurveillance and tumour immunoediting

In the early 20th century Ehrlich postulated that the immune system was able to recognise and eliminated cancer, a theory that was adapted into immunosurveillance by Thomas and Burnet in the 1950's [167]. Still, the immune system's ability to defend against cancer by recognising and eliminating tumour cells was debated for several decades. It was argued that indirect anti-tumour immunity might just be reached through the suppression of virally induced tumours or simply eliminating pathogens to resolve pro-tumourigenic inflammation [168]. However, human and murine studies from the 1980's offered compelling evidence for immunosurveillance controlling the growth of cancer. For example, Rag2$^{-/-}$ mice, which lack an adaptive immune system, were shown to have greater levels of spontaneous tumours [169], as did IFNγ-deficient mice [170]. Similarly, analysis of epidemiological data showed that humans who were immunocompromised, such as transplant recipients, were at a higher risk of developing non-viral associated cancers [171-173]. Observations were also made for higher levels of tumour infiltrating lymphocytes (TILs) being positively correlated with the improved prognosis for many cancers [174], including; skin

[175], breast [176], bladder [177], colon [178] and prostate [179]. From these data it was clear that the adaptive immune system could play an important role in preventing the development and progression of cancer.

With evidence for immunosurveillance, it was important to understand why cancer still developed in patients with functional immune systems. The three Es model of cancer immunoediting, described by Dunn and colleagues, defined distinct phases, elimination, equilibrium, and escape, which exist between the immune system and tumours [179, 180]. Elimination, the first phase of tumour immunoediting, comprises the immunosurveillance theory, whereby tumour cells are recognised and deleted by the immune system (**Figure 1.9**). The second stage, equilibrium, is entered when only partial elimination occurs, allowing for an equal balance between tumour cell death and regeneration. In both the first and second stages, there is a strong selection bias favouring the survival of less immune-susceptible tumour cells. The cancer is said to enter the final escape phase when the immune system is no longer capable of containing the net growth of tumour cells.

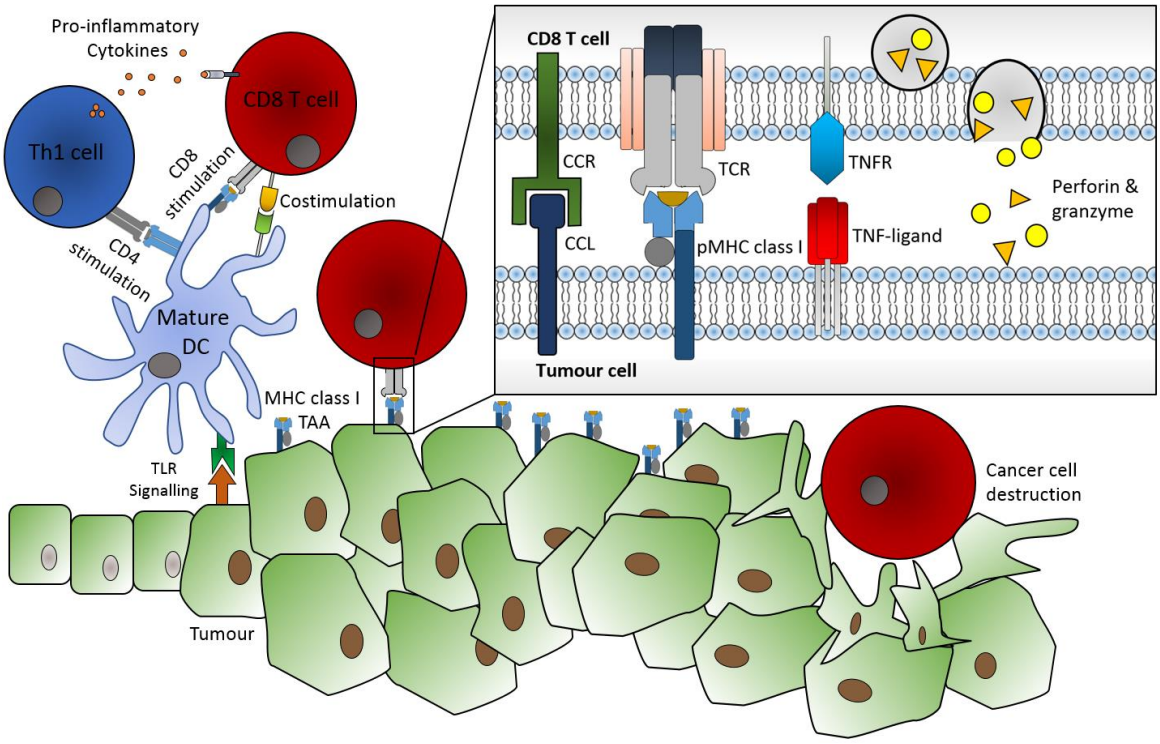

Figure 1.9: Tumour elimination by CD8 T cells. Tumour antigens are processed and presented by DCs, which are matured by TLR signals acquired from stressed or dying cells. The DCs MHC class II presentation of antigen allows for the stimulation of CD4 T cells, which express pro-inflammatory cytokines to aid the immune response. The MHC class I presentation of TAA on mature DCs, combined with co-stimulatory signals and inflammatory cytokines, can then

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activate tumour-specific CD8 T cells. Activated CD8 T cells traffic to a tumour site using chemokine ligands, such as the CCL family, whereupon the TCR can react with the TAA presented on the MHC class I molecule on a cancer cell. The CD8 T cell can then induce tumour death, by reacting with TNF-ligands on the tumour surface, such as TRAIL, and the release of cytotoxic molecules, perforin and granzyme. Additionally, T cell activation leads to the release of proinflammatory cytokines, such as IFNγ and TNFα, perpetuating the immune response and aiding the elimination of a tumour.

1.3.2 Tumour-associated antigens

An antigen expressed by a tumour can be defined as a tumour associated antigens (TAA), although the vast majority of these are poor targets due to their representation in healthy tissue facilitating immunogenic tolerance [181]. However, there are several different types of TAA that represent more feasible targets for T cells. Cancer-testis antigens, such as the MAGE antigen, are derived from proteins that are normally expressed in immune-privileged tissues and therefore do not have a pre-existing tolerance built against them [182]. Similarly, oncofetal antigens, such as carcinoembryonic antigen (CEA), come from proteins that are normally only expressed in fetal development and are therefore immunogenic. Additionally, differentiation antigens, such as MART-1, are derived from proteins only expressed in a small range of cells types. Neoantigens are produced from the mutated section of an otherwise normal protein may no longer be recognised as self-antigens. Additionally, mutations that cause normal proteins to be overexpressed can potently elicit an immune response due to the over-representation of a self-antigen. For cancer, both driving mutations, promoting tumour growth, and passenger mutation, having occurred but bearing no impact on cancer development, can both result in the presentation of immunogenic TAA. However, the immunogenic factor may be lost if a selective pressure is imposed by the immune system. This can occur through downregulating gene expression or disrupting the antigen processing and presentation pathway, allowing the tumour cells to evade recognition by T cells and avoid destruction [163, 183]. Finally, proteins from oncogenic viruses, such as HPV or EBV, can provide highly immunogenic foreign antigens and can be favourable targets due to their limited representation in other tissue.

1.3.3 Anti-tumour immune responses

Various immune cells are capable of aiding or directly orchestrating an anti-tumour response, including M1 macrophages, mature DCs, NK cells, B cells, CD8 T cells, and Th1, Th9, and Th17 CD4 T cells [184, 185]. For many cancers, however, CD8 T cells are believed to be the predominant mediator of anti-tumour immunity, strongly supported by the correlation between TIL infiltration and better disease prognosis [186]. Each stage of trafficking, recognition, and destruction by CD8

T cells can be hindered by tactics enlisted by cancer to attenuate the immune response. As such, simply introducing a large number of tumour-reactive T cells, through adoptive transfer or vaccination treatments, may be insufficient for total tumour eradication [187]. Indeed, T cells often fail in the context of large tumour burdens [188]. Many factors can contribute towards this failure, such as tumours disrupting antigen presentation, establishing vasculature barriers, and recruitment of immune-inhibitory leukocytes [186]. Understanding how anti-tumour immunity is both achieved and suppressed is critical for the future development of effective immunotherapies against cancer.

1.3.4 DC regulation of tumour immunity

As professional antigen presenting cells, DCs are vital for coordinating anti-tumour immunity by priming T cells. Typically, the uptake of TAA by a DC at the tumour site marks the start of the immune response [189, 190]. Provided that the DC collecting the antigen has also received maturation signals, it will be able to activate tumour-specific T cells within the tumour-draining lymph nodes or tertiary lymphoid organs featured within the tumour stroma [191]. Matured DCs can also stimulate NK cells that help drive anti-tumour immune responses [192]. The maturation signals for DCs, required for effective T cell priming, can come from various sources, including TLR ligands presented on necrotic tumours or factors derived from immunogenic cell death following chemotherapy treatment [193]. However, in the absence of such signals, non-matured DCs can present the TAA in a tolerising manner, promoting T_{reg} cells, inducing CD8 T cell anergy and suppressing the immune response [194-196].

There is considerable heterogeneity between the DC subsets, which include immature Langerhans cells, bone-marrow-derived myeloid DCs, and plasmacytoid DCs. The differentiation state is important for governing DCs contribution to tumour tolerance or clearance [197]. For example, the expression of $CD103^+$ is an important factor in allowing DC infiltration into tumours and priming a CD8 T cell response [198]. Most myeloid DCs in the tumour microenvironment display a partially mature phenotype, expressing high amounts of co-inhibitory molecules and immunosuppressive cytokines alongside intermediate levels of MHC class I and II and costimulatory markers [199]. Myeloid DCs isolated from human breast and lung cancer patients have been shown to be fairly ineffective at mediating T cell activation [199]. Interestingly, higher levels of dysfunction in myeloid DCs can be correlated with higher stages of cancer development.

Preventing DC maturation can be archived by several mechanisms and offers an effective way for the tumour to avoid immune destruction. Vascular endothelial growth factor (VEGF) was

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identified by Carbone and colleagues as a factor present in many tumours that suppresses DC function [200]. As well as limiting the maturation of DCs, VEGF induced the expression of PD-L1 on hematopoietic cells, a well-characterised checkpoint molecule that limits the proliferation and functional ability of T cells. The defects in DC maturation caused by the overexpression of VEGF in tumours are reversible upon treatment with the VEGF-blocking antibody (bevacizumab) [201]. However, many other tumour-derived soluble factors can suppress the maturation of DCs, including; TGFβ [202], IL-10 [203], macrophage colony-stimulating factor (M-CSF), and IL-6 [204, 205]. Additionally, high levels of hypoxia and lactic acid found within a tumour microenvironment promote indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2) expression by DCs, which are known to prevent T cell activation [206-208].

For many cancer patients, the mature DCs required to initiate an effective response is absent from the tumour site, and only low levels of tumour-reactive T cells are detectable [209, 210]. To take advantage of the ability of DCs to prime an immune response, a therapy has been developed to mature autologous DCs and load them with TAA *ex vivo* before administering them back into the patient. The DC vaccine 'Provenge' is FDA approved for the treatment of prostate cancer, conferring rather modest reductions in mortality [211]. Taken together, the evidence shows a dual role for DCs being able to promote or suppress anti-tumour immunity, dependent on maturation status.

1.3.5 T cell trafficking to the tumour site

There are many ways in which tumours can block or limit the recruitment of T cells. A major factor in this comes from the alterations to chemokine expression that orchestrate T cell trafficking. It is unsurprising that the tumours expressing high levels of T cell-attracting chemokines, such as chemokine (C-C motif) ligand 2 (CCL2), CCL3, CCL4, CCL5, chemokine (C-X-C motif) ligand 9 (CXCL9), and CXCL10, are seen to have the highest levels of T cell infiltration [212]. However, from these observations, it is not known whether the high levels of chemokines are present initially, or if T cell activation simply drives the chemokines' upregulation in a positive feedback loop. From murine models, it appears that only a few tumour-reactive T cells need to enter the tumour site to initiate an influx of non-specific T cells [213]. Additionally, there have been observations where the loss of EGFR-Ras signalling in murine melanoma cells leads to reduced CCL27, which resulted in reduced T cell accumulation and reduced anti-tumour immunity [214].

It is a common trait of cancer cells to disrupt post-translational modification pathways affecting factors such as glycosylation or cleavage of proteins. These changes can alter the expression or

function of the chemokines presented by cancer cells [215]. An interesting example of this comes from the nitrosylation of CCL2, which negates its ability to attract T cells while still attracting immunosuppressive MDSC to the tumour site [216]. Another example is the proteolytic processing of CXCL11 by tumour cells, which dramatically reduces the trafficking of $CXCR3^+$ effector T cells to the tumour site [217]. It is clear from this evidence that tumour-mediated suppression can be achieved in part through the control of chemokines that regulate T cell trafficking.

1.3.6 Passing the tumour vasculature

Even when T cells are able to accumulate in the tumour stroma, it is often difficult for them to infiltrate deeply into the tumour mass [218]. The tumour vasculature presents an active layer of endothelium, such as cancer-associated fibroblast (CAFs), that T cells must cross in order to recognise and destroy tumour cells [219]. Although there are still many unknown mechanisms within this area, it appears that the expression of adhesion molecules by the endothelium can be down-regulated by soluble tumour-expressed factors, prohibiting the passage of T cells and thereby creating an immune privileged site for a tumour. For example, the presence of VEGF in the tumour microenvironment suppresses the expression of T cell trafficking adhesion molecules on endothelium, such as ICAM-1 and vascular cell adhesion molecule (VCAM-1), even in the presence of TNFα [220]. Similarly, the expression of endothelin peptide ligands ER-1, which is found to be upregulated in a number of cancers, suppresses T cell adhesion to the endothelium, negating any upregulation of adhesion molecules attained from inflammatory cytokine stimulation [221]. In addition to their role as a physical barrier, endothelial cells can also express suppressive factors, such as FasL, TRAIL, PD-L1, TIM-3, IL-10, TGFβ, and PGE_2 [222, 223].

1.3.7 Encountering suppressive leukocytes

After overcoming the endothelial barrier, effector T cells will often have to compete against immunosuppressive leukocytes, including MDSC [224], T_{reg} cells [225], and M2 macrophages [226], which have also been recruited to the tumour site. These suppressive cells have a huge impact on anti-tumour immunity as their accumulation is correlated with a poorer prognosis for many cancers [227].

1.3.7.1 T_{reg} cell-mediated suppression

T_{reg} cells ($CD4^+$, $CD25^{High}$, $CD127^-$, and $FOXP3^+$) play a critical for maintaining tolerance towards self-antigens at peripheral sites, thereby preventing autoimmunity, and can be divided into two categories; natural T_{reg} (nT_{reg}) cells, derived from the thymus and maintained in the periphery by

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TGFβ, and inducible T_{reg} (iT_{reg}) cells, which are derived from naive CD4 T cells but are able to exert similar suppressive functions as nT_{reg} cells [228]. For both T_{reg} cell subtypes, the suppressive function is dependent on the expression of FOXP3. T_{reg} cells accumulate within a tumour, mediated by hypoxic conditions [229], cytokines such as IL-10 and TFG-β [230, 231], and chemokine ligands such as CCL22 and CCL28 [232, 233]. Typically, T_{reg} cells are restricted to a few dominant clones targeting tumour antigens [234, 235] with distinct TCRs from any other CD4 T cell subsets found within a tumour [234]. While T_{reg} cells are activated in a tumour-antigen specific manner, their suppressive capabilities are non-specifically mediated through the expression of inhibitory ligands, such as PD-L1 and CTLA4, and soluble factors, such as TGFβ and IL-10 [236]. These factors limit the ability of CD8 T cells to proliferate and produce effector molecules such as IFNγ and TNFα [237]. Furthermore, T_{reg} cells also interact with DCs, inducing them to express immunosuppressive factors such as IDO, IL-10, and TGFβ [225]. Paracrine IL-2 stimulation is necessary for the survival of T_{reg} cells. This is normally derived from activated CD8 T cells, therefore promoting T_{reg} accumulation in the event of an inflammatory immune response. Due to the high levels of CD25 expression, T_{reg} cells can suppress CD8 T cell indirectly by simply acting as an IL-2 sink [238]. Additionally, T_{reg} cells can directly kill CD8 T cells through the expression TRAIL [239] and granzyme B [232].

1.3.7.2 MDSCs

In cancer patients, MDSCs can often be found at relatively high levels within the peripheral blood and tumour site, where they mediate immunosuppressive functions. Originating from bone marrow, MDSCs are recruited to peripheral blood through BV8 and endocrine-gland-derived-VEGF [240]. MDSCs then traffic to the tumour site through chemokine interactions with CCL2, CXCL5, and CXCL12. Similar to T_{reg} cells, MDSCs are also attracted to hypoxic conditions that promote BV8 and CXCL12 expression [241]. Once at the tumour site, MDSC mediated suppression of T cells is achieved through the expression of TGFβ, IL-10, arginase I, and ROS [224]. Despite these pro-tumorigenic effects, it is also worth noting that MDSCs have phenotypic plasticity and can acquire the functionality of a tumour-rejecting monocyte or APC under the correct conditions, such as IL-12 and IFNγ stimulation [242].

1.3.8 Interaction of CD8 T cells with tumour cells

Even when tumour-specific T cells can overcome the vasculature, stoma, and immunosuppressive leukocytes, the cancer cells themselves possess several direct mechanisms for avoiding destruction. Cancers with a high mutational load generally have greater levels of immunogenicity [243], but a subsequent loss of immunogenic peptides are associated with a poorer clinical

prognosis [244]. For example, a selective advantage exists for tumours that downregulate classical HLA class I (HLA-A, HLA-B, and HLA-C) to reduce the ability of CD8 T cells to respond to TAA [245-247]. The increased risk of deletion by NK cells associated with this loss of expression of classical HLA molecules can also be compensated by cancer overexpressing HLA-G or HLA-E [248]. Tumour cells can also express surface molecules that directly kill T cells, such as TNF family genes FasL and TRAIL [249], or inhibit T cell function, such as PD-L1/2[250] and B7-H4 [251]. Soluble factors that suppress T cell function, such as TGFβ, IL-10, PGE_2, histamine, and adenosine can be expressed by tumour cells [249]. Furthermore, the consumption or enzymatic depletion of metabolic substrates in the tumour microenvironment can limit T cell activation [252]. For example, many cancer cells have been shown to express IDO [253], which catalyses tryptophan degradation. Tryptophan deprivation sensitises T cells to apoptosis, allowing IDO expression to be a powerful method for suppressing T cells in the tumour microenvironment [254, 255]. Finally, the hypoxic conditions and the accompanying drop in pH levels in the tumour microenvironment are known to inhibit effector T cell function [256]. Taken together, the strong selective pressure for avoiding immune detection, combined with the vast arsenal of immunosuppressive factors potentially available for a tumour to develop, it is unsurprising that many cancers enter and remain within the escape phase of immunoediting (**Figure 1.10**).

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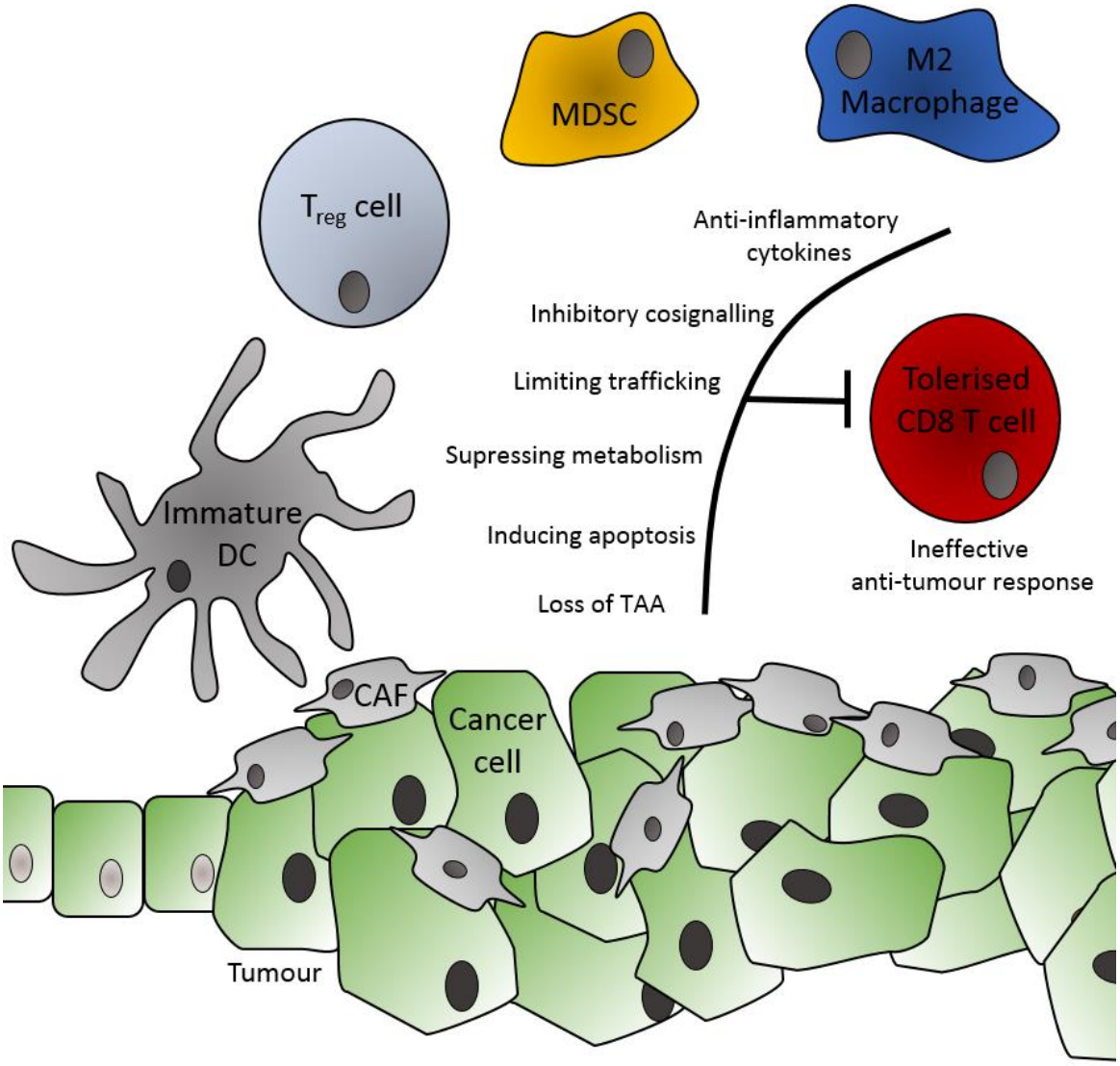

Figure 1.10: Immunosuppressive factors limit CD8 T cells anti-tumour response. A strong selective pressure exists for a tumour to mitigate destruction by CD8 T cells. This can be achieved through many mechanisms including, downregulation or loss of presentation for TAA, presenting ligands to induce apoptosis in T cells, creating an environment that suppress T cell metabolising, downregulating factors that promote T cell trafficking, establishing endothelium as a physical barrier to block T cell ingress, and the expression of inhibitory ligands and cytokines that suppress T cell stimulation. These factors may also be achieved indirectly through promoting the localisation of suppressive leukocytes, such as immature DCs, T_{reg} cells, MDCS, and M2 macrophages.

1.3.9 Restoring anti-tumour immunity

As discussed previously, tumours can evade the host immune response by exploiting physical barriers, immune suppression, tolerance, and leukocyte trafficking. Although overcoming these factors may appear to be a daunting task, recent developments have generated a great deal of

interest surrounding the restoration of effective anti-tumour immunity. Success stories from several immunotherapies have demonstrated that overcoming immunosuppressive conditions is achievable, with many advanced cancers displaying immunogenic targets that can be exploited through intervention. Successful results have stemmed from; Adoptive cell transfer (ACT) therapy with autologous T cell clones extracted from resected tumours and expanded *ex vivo* before transfer; ACT utilising endogenous lymphocytes transduced with a TCR or chimeric antigen receptor (CAR) to gain tumour specificity; Vaccinations with TAA or matured DCs loaded with TAA; Pharmacological inhibitors or mAbs to block immune-inhibitory molecules; Direct targeting of tumours or suppressive cell types by mAbs; Stimulatory mAbs targeting effector immune cells; And low doses of chemotherapy to disrupt vasculature, induce immunogenic cell death, or selectively deplete immunosuppressive cells. Due to the complexity surrounding tumour immunosuppression and the variety of immunotherapeutic options, utilising the optimal treatment is not always a straightforward choice. Current stratification relies on the tumour classification, indicators from previous studies, and preclinical evidence. While biomarkers can be somewhat limited, screening for TILs, TAA, and the presence of inhibitory factors have been utilised in some cases to decide on treatment options [257].

For tumours that contain TILs, it can be considered that factors preventing T cell accumulation are not completely insurmountable. Therefore, introducing a large number of tumour specific T cells to the system may be sufficient to provide an effective anti-tumour response. This rationale has been applied to the treatment of advanced melanoma and lymphoma, in which a significant proportion of advanced stage patients achieved objective clinical responses through ACT therapy [258].

Targeting tumours that do not present TILs may be inherently more difficult to treat with immunotherapy, as a tumour could be inaccessible or present a particularly immunosuppressive environment. Without tumour-reactive T cells at the tumour site, it is unlikely that a monotherapy such a checkpoint blockade therapy will promote an effective anti-tumour response. However, strategies synergising with checkpoint blockades to induce the rejection of previously non-immunogenic tumours exist such as; low dose IFNγ or histone deacetylases inhibitor to reverse epigenetic downregulation of MHC molecules [259, 260]; The use of whole-tumour antigen vaccination featuring all mutational epitopes [261]; And immunogenic cell death from chemotherapy or radiotherapy [262]. Taken together, blocking suppressive mechanisms while providing means of T cell stimulation represents a viable strategy for attaining potent anti-tumour responses.

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1.3.10 Cancer Immunotherapy

The treatment of cancer remains a challenging discipline, as patients often relapse following extensive treatments with surgery, radiation, and chemotherapy. While these conventional therapies are justified to extend the life of cancer patients, they often have associated risks and adverse side effects. Over the past decade, the potential for harnessing the power and specificity of immunotherapy has been realised, achieving clinical benefits in advanced cancer patients when conventional therapy has failed [263]. While it is possible to achieve durable remissions using these immunotherapies, many patients remain non-responsive. The mechanisms underlying immunotherapy resistance are incompletely understood and are likely to revolve around the mechanisms of immunosuppression. The development of more sophisticated immunotherapy strategies will require the evaluation of limiting factors and synergistic combinations to improve the range and efficacy of cancer treatment [264].

1.3.11 Monoclonal antibody therapy against cancer

As it is possible to generate mAbs targeting almost any surface protein, this technology has been utilised extensively in the progression of immunotherapy over the past few decades. The function of mAb is dependent on the choice of target, isotype, humanisation, and glycosylation status, but can serve one of several functions including, 1) Blocking survival signals on a cancer cell, 2) Blocking immune-inhibitory factors, 3) Driving immune-mediated tumour cell killing through antibody-dependent-cell-mediated-cytotoxicity, complement-mediated-cytotoxicity, or activating cellular phagocytosis, and 4) Immune stimulation by providing activatory signals [265].

Inadequate levels of positive stimulation or immunosuppressive factors often limit the ability of T cells to make an effective response against TAA. Therefore, mAbs have been used to directly stimulate costimulatory receptors or block suppressive molecules. A successful ‘checkpoint blockade’ strategy has been the use of Ipilimumab targeting CTLA-4, a negative regulator of T cell activation that binds to the B7 family of costimulatory molecules without providing activatory signals. Therapy with Ipilimumab has shown modest response rates, with 15% durable survival advantage, in phase III trials for advanced melanoma treatment [266, 267]. Targeting another checkpoint receptor, PD-1 and its ligand PDL-1 have also shown successful results in clinical trials. PD-1 is expressed on activated and exhausted T cells while PD-L1 can be expressed by tumour, stromal, and regulatory immune cells. Clinical studies have shown that αPD-1 and αPD-L1 therapy can achieve objective responses, even for solid cancers such as melanoma and lung cancer [268, 269]. Although there were some instances of PD-L1 negative tumours responding to this therapy, the best clinical responses were gained by the patients expressing the highest levels of PD-L1,

averaging approximately 50% objective responses of melanoma patients across several clinical trials [270]. Despite mAb therapy targeting PD-1 or CTLA-4 showing modest success across a range of cancers, many patients do not respond to treatment, displaying various resistance mechanisms including; T cell exhaustion, overexpression of caspase-8 in T cells, and MHC class I or II disruptions on cancer cells [271]. The efficacy of using costimulatory antibodies targeting CD40, GITR, OX40, CD137, ICOS, or CD27, or mAbs blocking other co-inhibitory ligands such as TIM-3, LAF-3, and BTLA, as monotherapies or in combination are currently being evaluated in preclinical and clinical settings [271]. Interesting progress has also been made in the use of bispecific antibodies facilitating targeted activation of T cells in the tumour microenvironment. The dual-specificity recombinant antibody structure allows for the binding of a TAA on the tumour surface while simultaneously stimulating T cells. For example, the combination of CD3 and CD19 on a bispecific antibody has shown some clinical success [272]. While this approach does not guarantee specificity of activation, tumour reactive T cell are more likely to accumulate within the tumour environment, and therefore become activated by this therapy. Taken together, the use of mAb immunotherapy has shown great potential for improving the therapy of certain cancers and their use is becoming increasingly prevalent, with more than 100 novel mAbs currently being evaluated in clinical trials [273].

1.3.12 Adoptive T cell therapy against cancer

While CD8 T cells have the ability to recognise and kill tumour cells, the immunosuppressive environment often limits the effector ability of TILs. The aim of ACT therapy is to administer a high number of functional tumour-specific T cells to generate sustained anti-tumour immune response in the cancer patient [274-276]. Recently, therapy has utilised advances in genetic engineering to facilitate functional improvements and endow specificity, achieving potently curative therapy for a range of advanced stage malignancies (**Table 1.2**).

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ACT	Year	Cancer histology	Target	Patients	ORs	Comments
TILs	1988	Melanoma [277]		20	55%	Original use TIL ACT
	2002	Melanoma [278]		13	46%	Lymphodepletion before cell transfer
	2011	Melanoma [279]		93	56%	20% CR beyond 5 years
	2014	Cervical cancer [280]		9	33%	Probably targeting HPV antigens
Transgenic TCR	2008	Melanoma [281]	NY-ESO-1	9	33%	High-affinity TCR clone
	2014	Leukaemia [282]	WT-1	12	100%	High risk for relapse; all patients cancer free at 27 month follow up
CARs	2011	Neuroblastoma [283]	GD2	11	27%	CR2 CARs into EBV-reactive cells
	2014	ALL [284]	CD19	30	90%	
	2014	Lymphoma [285]	CD19	15	80%	

Table 1.2: Selective examples illustrating developments in ACT. Adapted from a recent review of ACT by Steven Rosenberg and Nicholas Restifo [258]. OR; objective responses.

1.3.12.1 Development of the ACT protocol

Early studies by Rosenberg and colleagues provided evidence that certain tumours possess a concentrated source of lymphocytes [286]. For melanoma patients, these TILs were shown to respond to autologous tumour samples with the production of effector molecules [287]. Following these observations, murine models were used to demonstrate that the *ex vivo* culture of TILs in IL-2 could generate large numbers of cells, which, when transferred into tumour bearing mice, mediated the regression of liver and lung tumours [286]. These findings led to the use of autologous human TILs for ACT in a trial in 1988, which ultimately showed objective regressions for 11 out of 20 patients (55%) with metastatic melanoma [277]. This provided clear evidence that T cells could be utilised for the immunotherapy of cancer, but the response to ACT was often short-lived with regressions lasting 2 to 13 months. This issue was attributed to the fact that the transferred cells were rarely seen to have long-term persistence [258]. Improvements to the engraftment of transferred T cells was reported in 2002 with the use of non-myeloablative chemotherapy prior to ACT to generate a lymphopenic environment, reducing completion of homeostatic cytokines and decreasing the frequency of immunosuppressive T_{reg} cells, which was conducive to improved T cell persistence and resulting in several long-term tumour regressions [278]. As well as chemotherapy, total body irradiation can be utilised to generate a lymphopenic environment prior to ACT and is often used in preclinical murine models [47, 288]. This technique

also confers activation signals to the innate immune system, via microbes released from the radiation-damaged gut [288, 289].

Responses within early clinical ACT trials varied between patients, sometimes showing complete remission but in many cases only achieving transient responses or no response at all. It was clear that both the T cells intrinsic ability to persist and anti-tumour functionality were required for an effective and sustained response following ACT. However, a multitude of factors can contribute to ineffective responses, including; Terminal differentiation of the T cells following prolonged *ex vivo* culture in IL-2 [290]; Suboptimal affinity between tumour antigens and the TCR as a result of thymic selection and peripheral tolerance mechanisms eliminating the T cell clones that have strong responses [291]; Lack of cytokines or costimulation signalling to promote the survival or expansion of the transferred T cells [282]; Selective pressure forcing the outgrowth of antigen-negative tumour cells [180]; Or the abundance of immunosuppressive factors inhibiting the recruitment or function of the transferred T cells [292]. Further developments of ACT protocols and the genetic manipulation of T cells have allowed for the creation of T cells with improved survival and potent anti-tumour capabilities, increasing the range and efficacy that ACT can provide for cancer treatment. The use of genetically modified T cells gained notoriety following the incorporation of TAA-specific TCRs into autologous T cells, which provided a straightforward way of redirecting T cells towards a well-characterised tumour target (**Figure 1.11**) [293].

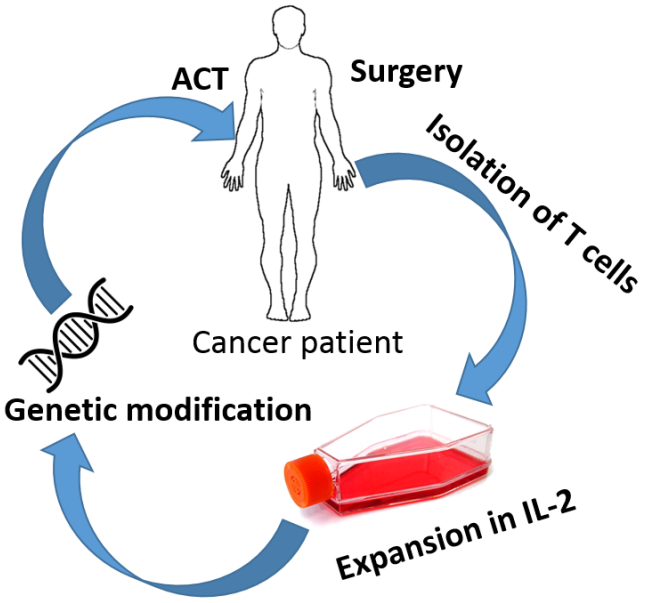

Figure 1.11: Schematic of ACT protocol. Following the surgical removal of the tumour mass, lymphocytes can be isolated from the tumour mass or PBMCs. The *in vitro* expansion offers a platform for the genetic manipulation of T cells prior to adoptive transfer. The T cells are then

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grown too large numbers before transferring back to patients, which have normally undergone lympodepletive chemotherapy and receive regular doses of IL-2 to aid the survival and expansion of cells *in vivo*.

1.3.12.2 Transgenic TCRs

Despite typical objective response rates often reaching above 50% for the treatment of advanced metastatic melanoma using *ex vivo* expanded TILs in the early 2000's, ACT remained largely unutilised. Many patients did not have resectable tumours, and for those who did, not all grew out lymphocytes. For the lymphocytes that were expandable, not all showed anti-tumour capabilities. In the cases where anti-tumour function was shown, the patients would then have to survive for months while the T cells were expanded to obtain a large number of cells necessary for therapy. This bespoke therapy was costly, requiring a large time commitment from specialised facilities and staff, and therefore there was a critical need to develop simpler methodologies for more universally applicable ACT therapies. From assessing TILs from hundreds of melanoma patient's samples, the common convergence of certain T cell clones recognising MART-1 and gp100 antigen were observed. This area was explored further through the development of murine tumour models, B16 melanoma expressing gp100, and transgenic pmel-1 mice, in which all CD8 T cells were reactive towards gp100, allowing for ACT to be investigated in preclinical models [294].

The theory that a common TAA could be targeted by a universal TCR for patients sharing the same HLA type was tested by Rosenberg and colleagues. In a clinical trial for the treatment of advanced melanoma, a CD8 T cell clone (DMF4) TCR, reactive to MART-1/HLA-A2, was transduced into peripheral blood T cells, which were then expanded in IL-2 *ex vivo* and transferred back into the patients. Compared to the isolation of TILs, this provided a quick and easy way to generate a large number of tumour-specific T cells, with generally lower proportions of terminally differentiated subsets. While the results were less impressive than the early trials using TILs, with only two out of seven patients achieving objective responses, this trial provided clear proof of principle for the use of TCR-transduced peripheral T cells for targeted treatment of cancer [295]. Moving forward from this point, it was important to address whether MART-1 was an optimal therapeutic target and to what extent the TCR affinity influenced therapy. Various MART-1 reactive clones were screened, with affinity defined as biophysical binding strength measured as the force required to separate the antigen from the target TCR by surface plasmon resonance, and higher affinity interactions (lower binding constant (KD) values) typically resulted in stronger levels of T cell activation [296]. The DMF4 clone, used for the first clinical trial, was shown to have a relatively low-affinity interaction with MART-1 (KD=170 μM) compared to other clones. Subsequently,

another clinical trial was designed using the highest affinity TCR clone, DMF5 (KD=43 μM). Upon treatment with endogenous peripheral T cells transduced with the DMF5 TCR, it was apparent that a much stronger immune response was occurring, to the point where off-tumour toxicity resulted in depigmentation and vitiligo [297]. MART-1-specific T cells were shown to infiltrate the skin, ears, and eyes, where they destroyed cells expressing reactive antigen such as healthy melanocytes expressing MART1. However, toxicities subdued after administering steroids. Following the first trial where no off-tumour toxicity was seen, it appeared that the increase in TCR affinity was responsible for the on-target off-tumour response. The high-affinity TCR (DMF5) also showed objective responses for 6/20 (30%) patients [297]. Furthermore, another high-affinity TCR for targeting gp100 went on to achieve similar levels of therapy and associated toxicities [297]. While the affinity of the TCR was a factor in the long-term efficacy of treatment, there was clearly risks associated with off-tumour reactivity when targeting self-antigens expressed in both tumour and normal tissue.

Following the use of MART-1 TCRs, there were transgenic TCRs therapies developed for the treatment of non-melanoma malignancies, targeting cancer testis antigen NY-ESO-1 and the oncofetal carcinoembryonic antigen CEA. Using retrovirus to deliver the NY-ESO-1/HLA-A2 (1G4) TCR into T cells prior to ACT, Rosenberg's group reported the objective responses from 11/18 (61%) synovial cell sarcoma patients and 11/20 (55%) melanoma patients [298]. Carl June and colleges have also run clinical trials using the NY-ESO-1 TCR, using a lentiviral transduction delivery system and achieving 16/20 clinical responses for myeloma patients in phase I/II trials [299]. In addition to melanoma, synovial cell sarcoma, and myeloma, there are clinical trials using NY-ESO-1 TCR to treat bladder cancer, breast cancer, neuroblastoma, lung cancer, ovarian cancer, and other metastatic malignancies [300]. Finally, the melanoma antigen MAGE-A3/HLA-A2 specific TCR has been used for the treatment of myeloma. While ACT treatment resulted in 5/9 clinical responses, a further three patients had severe off-target toxicity, resulting in two deaths due to an influx of transgenic T cells responding to MAGE-A12 expression in the brain [112].

1.3.12.3 Obtaining effective transgenic TCRs

A major hurdle for ACT is the identification TAA reactive TCRs that can offer a balance between effective tumour killing and limited off-tumour effects. An issue targeting TAA derived from non-mutated proteins is that endogenous TCRs is likely to have weak interactions and provide sub-optimal T cell stimulation. Mutagenesis techniques can be used to augment the TCRs affinity to pMHC. For instance, enhancing the ability of the CDR1/2 domain to the bind MHC molecule stabilises TCR interactions and enhances signalling, but this can be problematic due to permissive

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antigens triggering T cell activation [301, 302]. While modifications to the CDR3 are considered to give more antigen-specific enhancements, there have also been examples of serious off-tumour reactivity occurring [301, 302]. For example, the use of a MAGE-A3/HLA-A1 TCR, originally obtained from vaccinated patients was subject to affinity modulation through CDR3 mutation to create a high-affinity TCR and then used for the treatment of two patients, one with multiple myeloma and one with melanoma [303]. Both patients developed off-tumour cardiogenic shock and died within two weeks of receiving this ACT therapy. Although there was no MAGE-A3 detectable in the heart, only by screening of living heart tissues was a molecular mimic of MAGE revealed as antigen derived from the titin protein [303].

An alternative strategy for generating high-affinity TCRs is the use of mice that express human HLA, TCRα, and TCRβ loci but do not express the target antigen [304]. This approach runs a high risk of cross-reactive TCR being generated, as endogenous selections against many human antigens are not conserved in mice. However, this method has been utilised to generate high-affinity TCRs against the CEA for the treatment of metastatic colorectal cancer [305, 306]. When used in a clinical trial, objective tumour regression was observed in one out of three patients. However all patients developed colitis as a result of normal CEA expression in healthy tissues, and the trial was halted due to the associated toxicity. Regardless of how TCR affinities are enhanced, it is clearly of paramount importance to conduct rigorous safety screenings to assess the likelihood of cross-reactivity or off-tumour effects before moving into a clinical setting.

1.3.12.4 Targeting neoantigens

An approach to avoid off-tumour toxicity is targeting of neoantigens, as TCRs for these mutated epitopes are more likely to bypass tolerance mechanisms and therefore elicit higher affinity interactions. Indeed, there is evidence correlating the intra-tumour immune responses with the abundance of neoantigens [243]. However, many of the random mutations, from which neoantigens are derived, will not be oncogenic and can easily be lost from a tumour if a selection bias is imposed against these passenger mutations. Indeed, the neoantigen escape mechanism has been demonstrated in melanoma patients following TIL based ACT therapy [244]. Nevertheless, several common oncogenic driving mutations that are detectable in a significant fraction of patients could present ideal candidates for developing widely applicable neoantigen specific TCRs for gene therapy [307]. Investigations have shown that many of these common targets are however poorly immunogenic, which may a factor contributing to their relative abundance [308]. An alternative strategy could be to target multiple passenger mutations

simultaneously. While this is a viable strategy for effective immunotherapy, the logistics and costs currently associated with the generation and screening of a panel of neoantigen-TCR specific T cells for every patient represents a severely limiting factor [309]. Interestingly, work by Ton Schumacher and colleagues has demonstrated the possibility of using naive T cells from a donor, rather than an autologous source, for screening TCR reactivity against neoantigens [310]. As whole exome sequencing is rapidly becoming a quick and affordable process, it is likely that mutational screening would be achievable for many patients, yet further technical advances are required to accelerate the development of TCR screening and generation of TCR-transgenic T cells for therapies. Rather than attempting to optimise each individual TCR through mutagenesis, it is likely that universally applicable genetic modifications for improving the functionality of T cells will be of great benefit in this area.

1.3.13.5 Chimeric antigen receptor therapy

While transgenic-TCRs offer a potent form of ACT therapy, the treatment is inherently limited to patients possessing the correct HLA resection and is reliant on the tumours maintaining MHC expression (**Figure 1.12**). Opposingly, antibodies are not restricted by HLA and can recognise practically any surface molecule. An "immunoglobulin T cell receptor chimeric molecule" was first conceptualised in 1989, whereby the heavy and light chain variable regions of a mAb could be fused onto the constant region of the TCR and expressed by a T cell to trigger effector function following ligand binding [311]. Implementation of this led to the use of a single-chain fragment, encoding both heavy and light chains joined by a linker sequence, which could signal through an intracellular CD3ζ domain upon binding to cognate antigen, activating the transduced T cell [312]. This first generation of chimeric antigen receptors (CARs) was assessed in clinical trials targeting a folate receptor on ovarian cancer [313], carbonic anhydrase IX on renal cancer [314], and CD171 in neuroblastoma [315]. None of these clinical trials showed anti-tumour activity, which was not surprising given that the engraftment of CAR T cells was extremely poor. Positive results first came in 2008 for CAR T cell therapy targeting disialoganglioside G2D for paediatric neuroblastoma patients, which generated complete remission for 3/11 patients [283]. While the use of CAR T cells showed some potential, their lack of long-term persistence post adoptive transfer was a common problem within these early studies.

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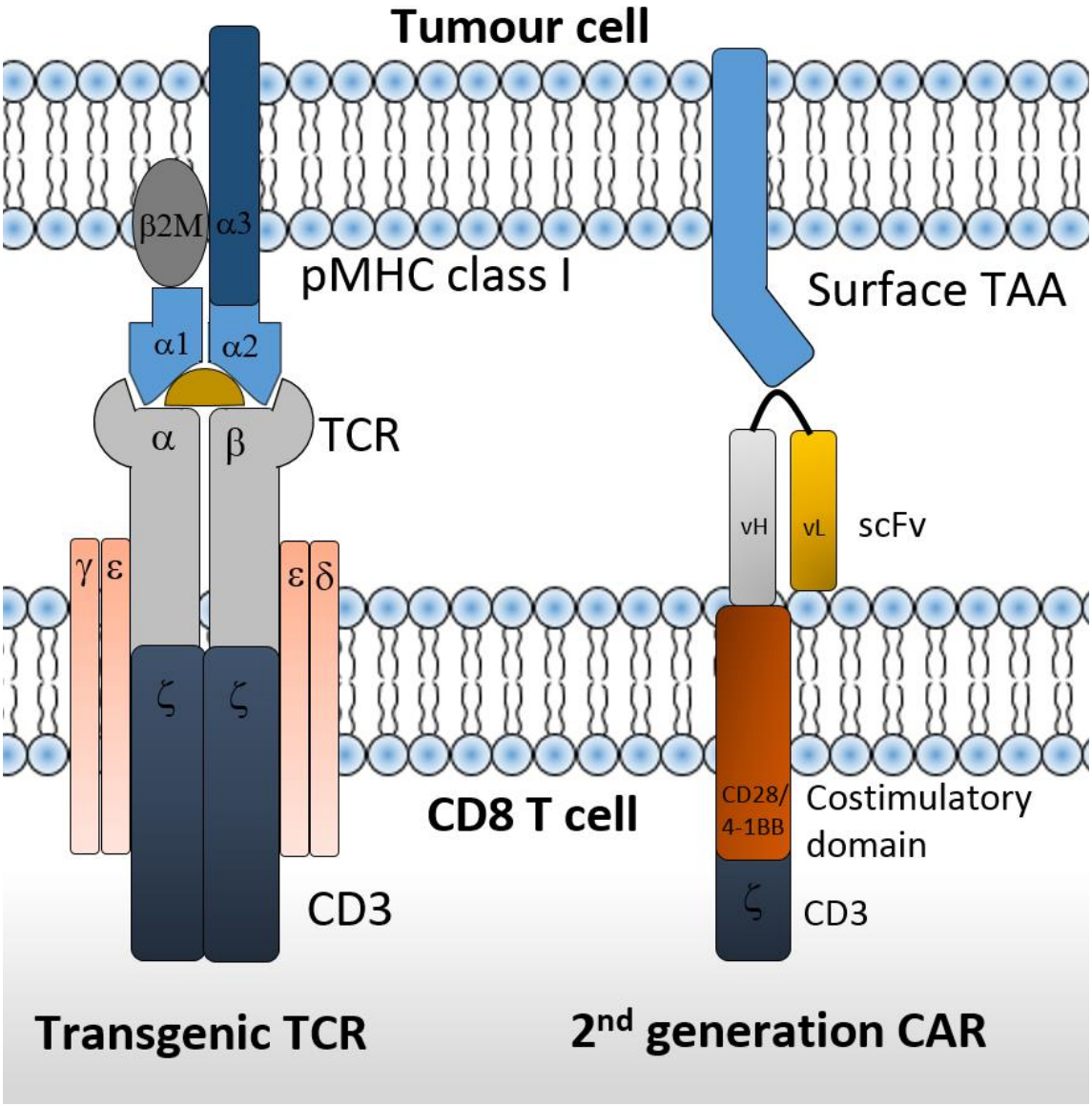

Figure 1.12: Structure of transgenic TCRs and CARs. A transgenic TCR comprises α and β TCR chains that form a regular TCR with endogenous CD3 chains and can interact with pMHC in a conventional manner. CARs utilise scFv antibody fragments to recognise non-MHC restricted surface proteins. The second-generation CARs possess an intracellular costimulatory domain as well as a CD3ζ chain to transduce activatory signals into the T cell upon ligand binding.

Improvements were made to the CAR design through the addition of costimulatory signalling domains. Modifications include the presence of an intracellular 4-1BB domain, which improved the survival of T cells through the upregulation of Bcl-xl, or the inclusion of an intracellular CD28 domain, enhancing the response to T cell signalling following stimulation [316, 317]. Second generation CARs were defined as containing one costimulatory domain, while third generation CARs contained several costimulatory regions (**Figure 1.12**). The most successful area of CAR therapy has been treating haematological malignancies by targeting CD19 with second or third

generation CAR T cells. For example, the treatment of advanced follicular lymphoma produced 6/8 clinical remissions, with 4/8 patients showing long-term depletion of all B cells, associated with high levels (>1%) of CAR T cells persistence within the PBMC at 10 days post transfer [318]. Similarly, use of CAR T cells has also shown great potential in the treatment of CLL [319] and multiple myeloma [320]. Furthermore, there are many ongoing clinical trials testing the ability of CAR T cells to target tumour-associated antigen on solid and haematological malignancies, targeting; HER2, GD2, cMET, MUC1, EphA2, GPC3, CD133, and CD171 [300].

1.3.13.6 Risk associated with ACT

Toxic side effects following ACT therapy are a serious concern due to off-tumour on-target reactivity or off-target cross-reactive responses. For example, the use of a third generation CAR T cell therapy targeting HER2, for treatment of a breast cancer patient, had fatal consequences due to the expression of HER2 antigen in lung epithelium [321]. There can also be systemic effects due to the high levels of inflammatory cytokines expressed or induced by the transferred T cells, such as IL-1, IL-2, IL-6, IL-12, TNFα, and IFNγ. These "cytokine storms" can cause various adverse effects including; Vascular leak syndrome, resulting in low blood pressure and oedema; Fever and malaise; And non-specific activation of innate and adaptive immune cells [322, 323]. While these side effects can be attenuated with corticosteroid treatment, this can reduce the function of the transferred T cells and therefore negate the intended anti-tumour response. It is difficult to assess the risk versus benefit when the level of tolerable toxicity varies between patients, especially as the patients that present with more toxic side effects regularly have higher chances of clinical responses. Future ACT treatments will likely include an inducible suicide gene, granting the ability to immediately attenuate the activity of transferred T cells in cases where side effects are considered life-threatening. For instance, systems have been developed to allow for a rapidly inducible caspase-9 suicide gene to effectively kill all transferred T cells [324].

1.3.13 Future developments in ACT

As well as the choice of the TAA to target, there are various other complexities sounding the optimisation of ACT that must be considered. The differentiation state of the transferred T cells along with the choice of additional genetic manipulations can be critical determinants of the cell's function and survival *in vivo*.

1.2.13.1 Ex vivo culture of T cells for ACT

The most common ACT protocol is as follows [258]. For the use of TILs, a tumour specimen is divided into multiple fragments or digested into a single-cell suspension and cultured with IL-2.

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The lymphocytes that outgrow a tumour within three weeks are then tested for tumour reactivity in co-culture assays. The reactive cultures are then expanded using αCD3, IL-2, and irradiated feeder cells. For transgenic TCR or CAR therapy, peripheral blood lymphocytes can be isolated and transduced, typically activating cells with αCD3 before applying retrovirus or lentivirus, then expanded in IL-2. For both TILs and transduced peripheral lymphocytes, two to three weeks of expansion in IL-2 can then generate up to 10^{11} tumour-specific T cells. Patients are lymphodepleted before ACT to aid engraftment [278], which is commonly achieved by chemotherapy with cyclophosphamide (60mg/kg) for two days and fludarabine (25mg/kg) for five days. After the *ex vivo* expanded T cells are transferred to the patient, IL-2 is administered at 720,000 UI/kg every 8 hours until it is no longer tolerated. While this protocol produces a large number of T cells for mediating anti-tumour immunity, often T cell survival is severely limited because the multiple rounds of stimulation and high levels of IL-2 exposure lead to increased levels of terminal differentiation.

Lower levels of T cell differentiation are correlated with an improved persistence post adoptive transfer in both clinical studies and preclinical murine models [325]. As such, T cell subsets expressing central memory provide an improved long-term anti-tumour response when compared to the use of more terminally differentiated effector subsets [279]. The factors that dictate T cell differentiation are therefore critical to consider tailor the subsets used ACT. These include the original source of lymphocytes (TILs or PBMCs), the method of *ex vivo* stimulation (antigen and irradiated cells or mAbs), the method of genetic modification (retroviral, lentiviral, or electroporation), and expansion of T cells (cytokine choice and culture duration). Indeed, compared to the conventional IL-2 expansion, exposing T cells to cytokines such as IL-7 and IL-15 [326] or IL-15 and IL-21 [327] results in lower levels of *in vitro* proliferation but helps maintain a greater proportion of central memory cells in the population. Promising clinical evidence for avoiding terminal differentiation comes from WT-1 specific T cell clones expanded in IL-21 for the treatment of high-risk leukaemia patients, demonstrating the long-term persistence of transferred cells in 3/4 patients with 2 of the patients showing long-term anti-tumour immunity [328].

1.2.13.2 Genetically manipulating T cells for ACT

Although genetic modifications with transgenic-TCRs or CARs can endow tumour specificity to *ex vivo* expanded T cells, ACT efficacy can be limited by many T cell-intrinsic factors that suppress the anti-tumour responses. Exploring how the genetic modification can enhance the function of T

cells for ACT has the translational potential for improving clinical treatments. For example, alongside the incorporation of a transgenic TCR therapy simply overexpressing CD3 improves expression of TCRs on the cell surface and thereby increases T cell sensitivity and anti-tumour immunity within a murine ACT model [329, 330].

A key factor influencing the survival of adoptively transferred T cells is the continued exposure to cytokines. A chimeric receptor can be used to translate extracellular inhibitory interaction into positive costimulation. For example, introducing a PD-1 extracellular domain fused to intracellular CD28 enhanced anti-tumour immunity in a preclinical ACT model [331]. Furthermore, an extracellular IL-4R domain fused to the IL-7 endodomain can reverse the inhibitory effects of tumour-derived IL-4, improving T cell survival, proliferation and anti-tumour immunity in a preclinical ACT model [332]. While the lymphopenic environment established in patients heightens the availability of endogenous IL-7 and IL-15, current ACT protocols have to utilise the administration of IL-2 to sustain terminally differentiated effector T cells, which consequently promotes the expansion of T_{reg} cells [333]. Within a murine ACT model, transducing cells to overexpress IL-7rα, has been shown to facilitate survival and proliferation of transferred cells, which can be enhanced with the exogenous administration of IL-7 selectively expanding transferred cells without promoting T_{reg} accumulation [334]. Alternatively, the overexpression of IL-2 or IL-15 by the transferred T cells can improve the *in vivo* expansion and anti-tumour activity in an antigen-dependent manner, as demonstrated in a preclinical model [324, 327]. The generation of T cells that overexpress IL-12 has been evaluated within a clinical setting. IL-12 cannot be delivered systemically due to severe toxicity observed in clinical trials [335]. However, in preclinical ACT models for the treatment of melanoma, leukaemia, or ovarian cancer, T cells overexpressing IL-12 were more resistance to TGFβ suppression and could mediate better antitumour immunity when low numbers of transferred T cells in the absence of lympodepletive preconditioning [336-339]. The use of a T cell activation inducible IL-12 gene (NFAT-IL-12) was shown to have less toxicity in preclinical models and has been used in a phase I clinical trial [340, 341]. Without having to administer IL-2, 11 out of 32 patients achieved objective responses in a dose-dependent manner [341]. Indeed, 63% of patients that received the high doses of cells ($>3x10^8$) experienced an objective response. However, in all cases, the persistence of the transferred T cells was severely limited and off-target toxicity associated with the IL-12 expression was observed.

The function of adoptively transferred T cells can also be improved by inhibiting the pathways or receptors associated with immunosuppression. Validated modifications include the inhibition of

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TGFβ type II receptor [342], the knockdown of FAS [342], and the knockdown of Cbl-b [343], promoting anti-tumour immunity in preclinical models of ACT. Using recent development in CRISPR/Cas9 systems, the disruption of PD-1 in human T cells was shown to enhance anti-tumour immunity in a preclinical NOD/SCID gamma (NSG) murine model [344]. Interestingly, this PD-1 disruption could be achieved alongside the removal of HLA-I and TCR and the introduction of a CAR, thereby showing progress in the development of a universal CAR T cell resistant to immune suppression [344]. The role of intrinsic factors that regulate T cell immunity against cancer has been explored by Wucherpfennig and college, who designed an *in vivo* screening technique by pooling libraries of shRNAs that were transduced into T cell prior to murine ACT [345]. The enrichment of T cells at tumour site was used as a functional readout and highlighted that improved accumulation was achieved through the knockdown of several proteins, including; Ppp2r2d (17.2 fold), Arhgap5 (15.7 fold), Alk (13.5 fold), Egr2 (10.2 fold), PTPN2 (7.4 fold) [345]. The knockdown of Ppp2r2 was then investigated further and shown to grant improved T cell proliferation, cytokine production and direct anti-tumour immunity in a preclinical ACT model [345]. Another study has shown that the knockdown Cish can improve responsiveness anti-tumour immunity in a murine model, attributed to improved signalling following TCR stimulation [346].

Overexpression of proteins that promote T cell function have also been explored in the context of ACT. For example, CD8 T cell function can be improved in the context of an immunosuppressive environment through the expression of constitutively active Akt, enhancing proliferation and cytokine production with sustained expression of NF-κB and anti-apoptotic factors [347]. Similarly, the overexpression of RAS homolog enriched in brain (RHEB), a positive regulator of mTORC1, promoted effector differentiation and improved anti-tumour immunity in a murine model [348]. The overexpression of the anti-apoptotic factor BCL-2 in adoptively transferred T cells can promote their survival, response to IL-2 stimulation, and antitumor immunity in preclinical ACT models [349]. Finally, the introduction of chemokine receptors can also aid in the trafficking of T cells to the tumour site. For example, in preclinical tumour models the overexpression of CXCR2 [350], CCR4 [351], or CCR2b [352], in T cells has been shown to improve anti-tumour immunity through mediating trafficking to CXCL1, TARC, and CCL2 respectively.

Taken together, it is clear that as well as redirecting antigen specificity, adoptively transferred T cells can benefit from further genetic modifications to enhance anti-tumour immunity. Elucidating mechanisms for improving T cell effector function, trafficking, and survival will enable ACT to become optimised and potentially personalised for a given cancer patient. However, as increasing

T cell function could enhance the danger of off-tumour reactivity, safety concerns need to be fully evaluated to minimise the risks of lethal toxicity occurring in patients. While several studies have detailed how the overexpression or inhibition of certain proteins impact T cell function, many potential targets remain to be validated in appropriate preclinical models assessing ACT efficacy against cancer.

1.4 Aims of the project

The aim of this project was to explore molecular mechanisms regulating CD8 T cell differentiation and function with a focus on the genetic modifications that would translate to the optimisation of ACT.

This entailed:

1. Chapter 3: Optimising preclinical murine ACT models' compatible with retroviral transduction of OT-I CD8 T cells; creating a retroviral vector to broaden the tools available for the purification of transduced cells and creating retroviral vectors expressing the gene or shRNA sequence of interest for use in the following investigations.
2. Chapter 4: Exploring the role of transcription factors FOXO1, FOXO3a and Eomes, in regulating the differentiation and function of CD8 T cells and elucidating if the constitutive activation of FOXO1 would provide a benefit to ACT therapy.
3. Chapter 5: Investigating the role of the phosphatases, PTPN2, PTPN7, and PTPN22, and the kinase, CSK, in regulating CD8 T cell function in the context of the preclinical ACT protocol.

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Chapter 2: Materials and methods

2.1 Reagents

2.1.1 Media and supplements for mammalian cell culture

Media compositions used for culture of mammalian cells are given below. Components were purchased from ThermoFisher unless otherwise stated.

Media	Components	Cells
Complete RPMI	RPMI media, FCS (10%) (Sigma-Aldrich), L-glutamine (200µM), sodium pyruvate (100µM), Penicillin-Streptomycin (50U/ml)	EL-4 and EG7
Complete DMEM	DMEM media, FCS (10%), L-glutamine (200µM), sodium pyruvate (100µM), Penicillin-Streptomycin (50U/ml)	Phoenix Eco
T cell media	RPMI media, FCS (10%), L-glutamine (200µM), sodium pyruvate (100µM), Penicillin-Streptomycin (50U/ml), 2-mercaptoethanol (50µM) (Sigma-Aldrich)	T cell

Additional reagents could be used to supplement T cells media, as described in the text.

Reagent	Application	Company
Concanavalin A (ConA)	CD8 T cell activation	Sigma
IL-2	CD8 T cell stimulation	PeproTech
IL-7	CD8 T cell stimulation	PeproTech
IL-12	CD8 T cell stimulation	PeproTech
IL-15	CD8 T cell stimulation	PeproTech

2.1.2 Media and supplements for bacterial cell culture

Media composition of the culture of bacteria, detailed below, could be constituted with or without the addition of agar (1.2%) (Melford).

Media	Components	Company	Cells
Lysogeny broth (LB)	Tryptone (1%), Yeast Extract (0.5%), Sodium Chloride (1%)	Melford	E. coli

Antibiotics were applied to the medium to select bacteria expressing a plasmid of interest.

Reagent	Working concentration
Ampicillin	100µg/ml
Kanamycin	50µg/ml

2.1.3 Buffers

The buffers, listed below, were prepared in distilled water, or PBS when stated, and corrected to a certain pH where appropriate. All chemicals components were Sigma-Aldrich unless otherwise stated

Buffer	Application	Components
Red cell lysis buffer	Red cell lysis	NH_4Cl (15.5mM), $KHCO_3$ (1mM), EDTA (0.01mM)
Protein solubilisation buffer (PSB)	Cell lysis	Tris (160mM, pH8), urea (6.4M), SDS (1.6%), bromophenol blue (0.08%)
Ripa buffer	Cell lysis	Tris (50mM, pH8), NaCl (150mM), NP-40 (1%), sodium deoxycholate (0.5%), SDS (0.1%)
Laemmli buffer	Protein loading dye	Tris (62.5mM, pH 6.8), SDS (2%), glycerol (10%), bromophenol blue (0.04%)
Transfer buffer	Western blot	Tris (112.5 mM), glycine (96 mM), ethanol (10%)
TTBS	Western blot	Tris (10mM), NaCl (10mM), Tween 20 (0.1%)
MOPS	Western blot	Tris base (50mM), SDS (3.47mM), MOPS (50mM), pH 7.7
Immunomagnetic purification buffer	MACS	PBS, BSA (0.5%), EDTA (2mM)
TBE	Gel electrophoresis	Tris borate (89mM), EDTA (2mM), pH 8.2
Annealing buffer	Oligopeptide annealing	Tris (10mM), EDTA (1mM), NaCl (50mM), pH 8.0

The list below details commercial buffers not prepared in-house

Buffer	Application	Components	Company
PBS	General	NaCl (13mM), KCl (2.7mM), Na_2HPO_4 (4.3mM), KH_2PO_4 (1.47 mM)	Severn Biotech
NuPage transfer buffer	Western blot		ThermoFisher
Trypsin EDTA	Cell culture	Trypsin, EDTA (0.25%)	ThermoFisher

2.1.4 Kits

Kits were used in accordance with the manufacturer protocol

Kit	Application	Company
QiaQuick Gel Extraction Kit	Purifying DNA from Gel	Qiagen
QIAprep Spin Miniprep Kit	Minipreps	Qiagen
Hi-Speed Plasmid Maxi Kit	Maxiprep	Qiagen
RNeasy Plus mini kit	RNA isolation	Qiagen

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2.1.5 Enzymes for molecular biology

All enzymes, listed below, were used following the manufacturer protocol

Enzyme	Application	Target sequence	Company
NotI	Restriction digestion	GC*GGCCGC	Promega
SalI	Restriction digestion	G*TCGAC	Promega
EcoRI	Restriction digestion	G*AATTC	Promega
XhoI	Restriction digestion	C*TCGAG	Promega
ClaI	Restriction digestion	AT*CGAT	Promega
HindIII	Restriction digestion	A*AGCTT	Promega
T4 DNA Ligase	DNA ligation		Promega
Go Taq polymerase	PCR		Promega
Pfu	PCR		Promega

The buffers used for molecular biology are listed below.

Buffer	Application	Company
Buffer D	Restriction digestion	Promega
Buffer E	Restriction digestion	Promega
Buffer H	Restriction digestion	Promega
MultiCore buffer	Restriction digestion	Promega
10x Ligase buffer	DNA ligation	Promega
10x Pfu reaction buffer	PCR	Promega

2.1.6 Primers for PCR, qPCR, and sequencing

All oligonucleotides, listed below, were custom synthesised by ThermoFisher.

Primers	Sequence
pLXSN 5'	CCCTTGAACCTCCTCGTTCGACC
Thy1.1 (A) Forward	AAATCGATTCACAGAGAAATGAAGTCCA
Thy1.1 (A) Reverse	TTAAGCTTGGCCACAACCATGGGTCTTTTCTGCAGT
Thy1.1 (B) Forward	AAATCGATTCACAGAGAAATGAAGTCCAGGGCTTGGAG
Thy1.1 (B) Reverse	TTAAGCTTGGCCACAACCATGGGTCTTTTCTGCAGT

TaqMan® Gene Expression Assays for qPCR were ordered from ThermoFisher.

Probe	Assay ID
IL-2	Mm00434256_m1
IFNγ	Mm00801778_m1
TNFα	Mm00443258_m1
B2M	Mm00437762_m1

2.1.7 Oligonucleotide sequences for shRNA inserts

All oligonucleotides, listed below, were custom synthesised by ThermoFisher and inserted into the pMKO.1 GFP vector.

shRNA	Sense oligo	Antisense oligo
PTPN22-A	CCGG**CCGATGAGGATTCCAGTTATA**CTCGAGTAT AACTGGAATCCTCATCGGTTTTTG	AATTCAAAAA**CCGATGAGGATTCCAGTTATA**CTCGAGTA TAACTGGAATCCTCATCGG
PTPN22-B	CCGG**CCGTGCAAACTTCTTCTACTA**CTCGAGTAGT AGAAGAAGTTTGCACGGTTTTTG	AATTCAAAAA**CCGTGCAAACTTCTTCTACTA**CTCGAGTA GTAGAAGAAGTTTGCACGG
PTPN22-C	CCGG**CGGACCAAATCAACTCCCTTT**CTCGAGAAA GGGAGTTGATTTGGTCCGTTTTTG	AATTCAAAAA**CGGACCAAATCAACTCCCTTT**CTCGAGAA AGGGAGTTGATTTGGTCCG
PTPN22-D	CCGG**CGGCTAAATCAAGCCCTTCTT**CTCGAGAAG AAGGGCTTGATTTAGCCGTTTTTG	AATTCAAAAA**CGGCTAAATCAAGCCCTTCTT**CTCGAGAA GAAGGGCTTGATTTAGCCG
PTPN7-A	CCGG**TGGTCTGACCTTGGTCAAATC**CTCGAGGAT TTGACCAAGGTCAGACCATTTTTG	AATTCAAAAA**TGGTCTGACCTTGGTCAAATC**CTCGAGGA TTTGACCAAGGTCAGACCA
PTPN7-B	CCGG**ACCACACTTTGGCCCTATATG**CTCGAGCATA TAGGGCCAAAGTGTGGTTTTTG	AATTCAAAAA**ACCACACTTTGGCCCTATATG**CTCGAGCA TATAGGGCCAAAGTGTGGT
PTPN7-C	CCGG**AGCTGGAGTGCTGGCTTATTT**CTCGAGAAA TAAGCCAGCACTCCAGCTTTTTG	AATTCAAAAA**AGCTGGAGTGCTGGCTTATTT**CTCGAGAA ATAAGCCAGCACTCCAGCT
PTPN7-D	CCGG**CACCACACTTTGGCCCTATAT**CTCGAGATAT AGGGCCAAAGTGTGGTGTTTTTG	AATTCAAAAA**CACCACACTTTGGCCCTATAT**CTCGAGAT ATAGGGCCAAAGTGTGGTG
PTPN2	CCGG**CCTGTCTTGTTCTGATGGAAA**CTCGAGTTTC CATCAGAACAAGACAGGTTTTTG	AATTCAAAAA**CCTGTCTTGTTCTGATGGAAA**CTCGAGTT TCCATCAGAACAAGACAGG
Csk-A	CCGG**CAAGAAGTACGAATCTTATTT**CTCGAG AAATAAGATTCGTACTTCTTGTTTTTG	AATTCAAAAA**CAAGAAGTACGAATCTTATTT**CTCGA GAAATAAGATTCGTACTTCTTG
Csk-B	CCGG**CGGTACAGAATGTATTGCCAA**CTCGA GTTGGCAATACATTCTGTACCGTTTTTG	AATTCAAAAA**CGGTACAGAATGTATTGCCAA**CTCG AGTTGGCAATACATTCTGTACCG
Csk-C	CCGG**AGTACCTGGAGGGTAACAATT**CTCGA GAATTGTTACCCTCCAGGTACTTTTTTG	AATTCAAAAA**AGTACCTGGAGGGTAACAATT**CTCG AGAATTGTTACCCTCCAGGTACT
Csk-D	CCGG**GCAGTCAAGTGCATCAAGAAT**CTCGA GATTCTTGATGCACTTGACTGCTTTTTG	AATTCAAAAA**GCAGTCAAGTGCATCAAGAAT**CTCG AGATTCTTGATGCACTTGACTGC
Scrambled	CCGG**ATGCCTATTCGTGATATCGGT**CTCGAGACC GATATCACGAATAGGCATTTTTTG	AATTCAAAAA**ATGCCTATTCGTGATATCGGT**CTCGAGAC CGATATCACGAATAGGCAT

2.1.8 Antibodies used for flow cytometry

Expression of surface and intracellular proteins were analysed by flow cytometry. Antibodies used are listed below. Additionally, fluorochrome labelled SIINFEKL H-2K^{b} tetramer (produced in-house by the protein core facility, CSU, University of Southampton) specific for the OT-I TCR, was utilised for flow cytometry.

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Antibody	Clone	Isotype	Company
CD8	53-6.7	Mouse IgG2a	eBioscience
CD4	GK1.5	Rat IgG2b	eBioscience
CD62L	MEL-14	Rat IgG2a	eBioscience
CD127	A.7R34	Rat IgG2a	eBioscience
CXCR3	CXCR3-173	Mouse IgG	eBioscience
CD43	eBioR2/60	Rat IgM	
IL-2	JS6-SH4	Rat IgG2b	eBioscience
Eomesodermin	DAN1/ 1MAG	Rat IgG2a	eBioscience
CD44	IM7	Rat IgG2b	eBioscience
CD25	PC61.5	Rat IgG1	eBioscience
Granzyme B	NG3B / GB11	Rat IgG2a / Mouse IgG1	eBioscience / Cell Signalling
IFNγ	XMG1.2	Rat IgG1	eBioscience
TNFα	MP6-XT22	Rat IgG1	eBioscience
T-bet	eBio4B10	Mouse IgG1	eBioscience
CD45.1	A20	Mouse IgG2a	eBioscience
CD69	H1.2F3	Armenian hamster IgG	eBioscience
FOXO1	L27	Rabbit polyclonal	Cell Signaling
FOXO3a	75D8	Rabbit polyclonal	Cell Signaling
CD43	1B11	Rat IgG2a	BioLegend
KLRG1	2F1	Mouse IgG1	eBioscience
Thy1.1	HIS51	Mouse IgG2a	eBioscience
Mouse IgG2a Isotype	eBM2a	Mouse IgG2a	eBioscience
Rat IgG2a Isotype	eBR2a	Rat IgG2a	eBioscience
Mouse IgG Isotype	P3.6.2.8.1	Mouse IgG1	eBioscience
Rat IgG2b Isotype	eB149/10H5	Rat IgG2b	eBioscience
Rat IgG1 Isotype	eBRG1	Rat IgG1	eBioscience
Anti-rabbit IgG	Poly4064	Donkey polyclonal	BioLegend
FCγR II/III	2.4G2		In-House

2.1.9 Antibodies used for Western blot

The antibodies listed below were used to detect and quantify proteins by Western blot.

Antibody	Dilution	Isotype	MW (kDa)	Company
PTPN2	1:5000	Mouse	45	R&D Systems
PTPN7	1:5000	Mouse	40	Abnova
PTPN22	1:1000	Rabbit	98	BioLabs
CSK	1:5000	Rabbit	50	Abcam
Actin	1:200	Goat	42	Santa Cruz Biotechnology
αMouse IgG - HRP	1:5000	Sheep		GE Healthcare
αRabbit IgG - HRP	1:5000	Donkey		GE Healthcare
αGoat IgG - HRP	1:2000	Donkey		Santa Cruz Biotechnology

2.1.10 Therapeutic antibody

The antibody, listed below, was used for the immunisation of mice at the dose described in the text.

Antibody	Clone	Company
Anti-CD40	3/23 [353]	In-house (Antibody and Vaccine Group, University of Southampton)

2.2 Mice

Wild-type C57BL/6 mice and OT-I transgenic mice, in which all CD8 T cells possess H-2K^{b}/SIINFEKL–specific TCR ($OVA_{257-264}$), were purchased from Charles River and bred within the in-house biomedical research facility. Mice were aged between 8-12 weeks old at the start of tumour experiments. All animals were housed in the BRF, and all experiments were conducted in accordance with the University of Southampton and the UK Home Office guidelines and approval.

2.3 Tissue culture

2.3.1 Mammalian cells

The Phoenix Ecotropic retroviral packaging cell line was purchased from ATCC and cultured below 90% confluency in complete DMEM medium without antibiotics. Trypsin EDTA (ThermoFisher) was applied for five minutes to detach the adherent cells from the plate.

The EG7 tumour cell line was purchased from ATCC and cultured in complete RPMI medium supplemented with G418 (geneticin) (ThermoFisher) at 400µg/ml selection. 24 hours before the EG7 cells were used for an *in vivo* experiment, cells were washed and cultured without antibiotic selection.

The EL4 tumour cell line was purchased from ATCC and cultured in complete RPMI medium. Cells were counted using a Coulter Industrial D Cell Counter (Beckman Coulter). Viability was measured by diluting cells with Trypan Blue (Sigma-Aldrich) 1:1 and manual count using a haemocytometer.

2.3.2 Bacterial cells

Chemical competent E. coli, One Shot Top10 (ThermoFisher) or One Shot STBL3 (ThermoFisher), expressing plasmids of interest were cultured with antibiotic selection on LB at 37^{o}C.

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2.4 Molecular biology

2.4.1 Plasmids

The pMKO.1 GFP vector was a gift from William Hahn purchased from Addgene (# 10676). The pMKO.1 puro vector was a gift from Bob Weinberg purchased from Addgene (# 8452). The pMP71 vector was a kind gift from Hans Stauss. The MSCV-IRES-Thy1.1 (pMiT) vector was a gift from Anjana Rao purchased from Addgene (# 17442).

Preparation of FOXO1 AAA and FOXO3a AAA plasmids on the pMP71 backbone was performed by Dr Sarah Buchan (University of Southampton).

2.4.2 Agarose gel electrophoresis

Agarose powder (ThermoFisher) was mixed with TBE buffer and microwaved until fully dissolved. Typically, 2% agarose was used for samples < 500 bp, while 1% agarose was used for the separation of larger fragments. Ethidium bromide (Fluka) was added ($5x10^{-5}$%) to the dissolved agarose. The DNA samples were mixed with 6x orange loading dye (Fermantas) and loaded onto the gel alongside an appropriate ladder (100 bp or 1 kb O'gene ruler (Fermatas)). The gels were run at 70-120 volts and analysed using a UV transilluminator Gel doc (Bio-Rad).

2.4.3 Restriction digestion of plasmids

In a volume totalling 20µl, DNA (0.1-1.5µg) was digested with restriction enzyme/enzymes (5-10U/µg - Promega) in the appropriate buffer to achieve 75-100% activity in the 2 hours incubation (37^{o}C). The total concentration of glycerol did not exceed 10% of the reaction mixture.

2.4.4 Gel purification of digested plasmid fragments

After restriction digestion of the plasmids, DNA fragments were separated by agarose gel electrophoresis. The lanes for purification were removed before guider lanes were visualised with UV and used to mark where the fragment of interest had migrated. The section of gel containing the digested fragment was then purified using a gel extraction kit.

2.4.5 Oligopeptide annealing

Complementary oligopeptides were mixed (1:1), diluted (1pmole/µl) in an annealing buffer, heated at 95^{o}C for 5 minutes and allowed to cool slowly to room temperature.

2.4.6 DNA ligation

DNA fragments or annealed oligopeptides were mixed with a linearised vector (1:3 or 1:1000, vector to insert ratio, respectively) and incubated with T4 DNA ligase (1u) for 3 hours at room temperature. This ligation mixture was then used to transform chemically competent bacteria.

2.4.7 Bacterial transformation

Chemically competent E. coli were transformed by the standard chemical transformation procedure. Briefly, 1-10ng of plasmid was added to E. coli, thawed on ice for 45 minutes, heat shocked (42^{o}C) for 30 seconds then cultured for an hour with SOC media (250µl). The transformed bacteria were then spread on an LB agar plate with antibiotic resistance and incubated overnight (37^{o}C) to allow single colonies to form.

2.4.8 Plasmid production in E. coli

For Minipreps, a single colony of E. coli was inoculated into 5ml LB with antibiotic selection and cultured for 12 hours (37^{o}C, 200 rpm) before use with the Miniprep Kit. For Maxiprep, a single colony of E. coli was inoculated into 5ml LB (100µg/ml ampicillin) and cultured for 8 hours. Between 1-5ml of this culture was then inoculated into 150ml LB with antibiotic selection for 12 hours, which could then be used with the Maxiprep kit.

2.4.9 Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify specific sequences of DNA for both cloning and analytical purposes. Under sterile conditions, primers (3.2pM) were combined with DNA (100ng), dNTP (10mM) GoTaq DNA polymerase (Promega; 0.1u) and GoTaq Buffer (Promega). The DNA was then amplified using a PTC-100 (MJ research) thermocycler. Products were assessed and purified by electrophoresis on an agarose gel.

2.4.10 qPCR

A qPCR protocol was used for the quantification of messenger RNA. Firstly, the RNA from a population of $1x10^{6}$ - $5x10^{6}$ cells was extracted using an RNeasy Mini kit (Qiagen), following the manufacturer's instructions. The RNA was stored at -80^{o}C until used in a reverse transcription reaction to generate cDNA. For this reaction, 200ng of RNA was used with the SuperScript™ III Reverse Transcriptase kit (ThermoFisher) following the manufacturer's protocol. The cDNA was then stored at -20^{o}C until use in qPCR. The Platinum® Quantitative PCR SuperMix-UDG kit (ThermoFisher) was used for qPCR reactions, following the manufacturer's protocol. Briefly, 10ul Platinum® Quantitative PCR SuperMix-UDG, 1ul of qPCR primer, 0.5ul cDNA sample and 8.5ul was used per reaction. The samples, loaded on a 96 well plate was used in (machine), and the

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following protocol was used: 50°C for 2 minutes hold (UDG incubation) 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. The figures were generated using the capture software. ΔCT was calculated by taking the cycle threshold of the control gene (B2M) away from the cycle threshold of the gene of interest. ΔΔCT was calculated as 2(-(X-Y)-(A-B)): X = the gene of interest in the treatment sample, Y = Control gene in the treatment sample, A=Gene of interest in a control sample, B= Control gene in control sample. The fold change in $2^{\Delta CT}$ relative to the control was used to compare the mRNA levels for different treatment conditions.

2.4.11 Sequencing

Big-dye reaction mix (ThermoFisher) was used for the sequencing and validation of shRNA oligonucleotide inserts within the pMKO.1 GFP vector with the primer pLXSN 5', specific for the murine stem cell virus promoter, following the manufacturer's protocol. The reaction consisted of 5x sequencing buffer (2µl), Big-Dye reaction mix (2µl), primer (1µl), target DNA (0.5µg) and made up to a final volume of 10µl with distilled water. The reaction was carried out in a thermocycler (25 cycles: 94°C – 10 seconds, 50°C – 5 seconds, 60°C – 4 minutes). The products were then precipitated using 2µl NaAc (3M) and 48µl ethanol (100%) and then centrifuged at maximum speed for 30 minutes (4°C). After removing the supernatant, the sample was washed with 180µl ethanol (70%) and centrifuged for a further 10 minutes. The supernatant was removed and the sample was air dried at 37°C before adding 10µl formaldehyde to denature the sample. The samples were run on an automated sequencer (Applied Biosystems 2120 XL genetic analyser). The data generated were then analysed with SeqMan Pro (Lasergene 12) software.

2.4.12 Western blot

Cell samples were lysed in PSB or Ripa buffer, the latter of which allowed for the total protein to be quantified by a BCA protein assay (ThermoFisher) following manufacturer's protocol. DTT (50mM) and Laemmli buffer were added to sample lysates. Typically, 20-50µg of protein was loaded onto a precast 10% Bis-Tris NuPage Gel (ThermoFisher), and separation was carried out at 100V-150V in MOPs buffer). Proteins were then transferred to a PVDF membrane (Immobilon), using Nupage transfer buffer at 100V for 1 hour. Ponceau Red staining was visualised using a Gel doc (Bio-Rad) with white light, to assess total protein levels on the membrane, which was then washed with western blot buffer and blocked with milk (5% in TTBS). Incubation with primary antibody diluted in milk (5% in TTBS) was performed for one hour at room temperature or overnight at 4°C. The membrane was washed four times with TTBS before adding secondary antibody was added, diluted in milk (5% in TTBS) for one hour at room temperature. The membrane was washed four times with TTBS before applying ECL Western blotting substrate

(ThermoFisher or GE Healthcare) following the manufacturer's protocol. The bands were visualised by exposing the membrane to Amersham Hyperfilm ECL (GE Healthcare) within a cassette. Quantification of bands was assessed using ImageJ software. For the comparison of protein levels then samples were normalised against the Ponceau or Actin.

2.4.13 Production of retrovirus

$1.5x10^5$ Phoenix Eco cells were seeded in 3mls of media within a p6 well and cultured for three days (reaching 70% confluency), transfected with FuGENE HD (Promega) following the manufacturer's protocol. Briefly, the retroviral plasmid of choice (4µg) and pCLEco (4µg), a gift from Inder Verma (Addgene plasmid # 12371) to enhance retrovirus packaging, were incubated with FuGENE HD for 10 minutes before being applied to a p6 well of the Phoenix Eco cells. The supernatant was replaced 24 hours later with T cell media. One day later (48h post-transfection) the viral supernatant was collected and used for the retroviral transduction of CD8 T cells (see **Chapter 2.7.4**). The transfected Phoenix Eco cells were harvested and to assess the transfection efficiency by flow cytometry.

2.5 Flow cytometry

2.5.1 Surface staining

Typically, $0.2\text{-}1x10^6$ cells were added to a FACs tube for analysis and washed with 3ml of PBS 0.1% BSA. The T cell samples were then blocked with 100µl of 2.4G2 (10µg/ml) for ten minutes (4^oC). 100µl of fluorochrome labelled mAbs, (1-10µg/ml), diluted in PBS 0.1% BSA, were used to stain the cells for 30 minutes (4^oC) in the dark. The cells were washed with 3ml of PBS 0.1% BSA and then used for further intracellular staining when indicated. Data collected with the BD FacsCanto II were and analysed using the BD FACSDiva (V6.1.2) software (BD Bioscience). Alternatively, data collected with the FACSCalibur were analysed with Cellquest Pro (BD Bioscience). The histogram overlays were produced using FCS Express (V.3) software (De Novo Software)

2.5.2 Intracellular/nuclear staining

Surface stained samples were fixed with 300µl of the Foxp3 Fixation/Permeabilisation solution (eBioscience), and each sample was incubated according to the manufacturer's protocol. The samples were washed with 2ml of Permeabilisation buffer (eBioscience). Antibodies were diluted in 100µl of Permeabilisation Buffer and added to each sample before a 30-minute incubation (room temperature). The cells were washed in Permeabilisation buffer and analysed by flow cytometry.

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For the analysis of intracellular cytokines, staining was performed with the BD Cytoperm/Fixation kit (BD Bioscience) following the manufacturer's protocol. Cells were fixed with BD Fixation Buffer (100µl) for 15 minutes at room temperature in the dark. BD Permeabilisation buffer (100µl) was added, and the sample was washed. The cells were then stained with intracellular antibodies, diluted in BD Permeabilisation buffer, for 30 minutes at room temperature. The samples were washed again with BD Permeabilisation buffer then resuspended in PBS (0.1% BSA) before analysis was carried out by flow cytometry

2.6 Cell subset purification

2.6.1 Immunomagnetic sorting

Cell populations (T cells, CD45.1$^+$, Thy1.1$^+$) were enriched using a MACS (Miltenyi Biotec) selection kits, or using primary fluorochrome or biotin labelled antibodies and then using secondary anti-primary-antibody conjugated to magnetic beads. This protocol was carried out following the manufacturer's instructions with LD or LS columns. The purity and yield were assessed by flow cytometry.

2.6.2 FACS purification

For the purification of transduced CD8 T cells 24 to 48 hours post-transduction, cells were stained with a CD4 antibody. The cells were then run on a FACS Aria II (BD Bioscience) selecting GFP$^+$ CD4$^-$ cells using the purity setting.

2.7 *In vitro* experiments

2.7.1 CD8 T cell activation and differentiation assay

The OT-I splenocytes were isolated using a cell strainer, washed in PBS and treated with red cell lysis buffer. The splenocytes were resuspended (1.5x10^6/ml) in T cells media and activated with either ovalbumin-derived peptide $OVA_{257-264}$ (SIINFEKL; herein referred to as SIIN), ConA. Additionally, activation was augmented using various cytokines as described in the text.

For the *in vitro differentiation assay,* 48 hours after activation the cells were washed and resuspended (2x10^5/ml) in T cells media the presence of IL-2 (10ng/ml) or IL-15 (10ng/ml). The cells were cultured for 72 hours, during which cells were resuspended back to 2x10^5/ml in T cell media containing the cytokines daily.

2.7.2 CD8 T cell killing assay

Purified transduced OT-I cells were expanded for five days in IL-2 (10ng/ml) refreshing cytokine daily. Following the expansion, the transduced cells were cultured for four hours with a 1:1 mix of EG7 target cells stained in CFSE (ThermoFisher) (10μM) and EL4 cells non-target cells stained with CTV (ThermoFisher) (10μM). The staining process was performed following manufactures protocols. Following this incubation, the ratio of EG7 to EL4 was determined by flow cytometry by gating on the $CD45.1^-$ population

2.7.3 CD8 T cell cytokine production assay

At least three days after initial activation, intracellular cytokine production was assessed by *in vitro* restimulation of the CD8 T cells. 1-2x10^5 OT-I T cells were added to a U bottom p96 well with GolgiPlug™ (1:1000) (BD Bioscience) and restimulated with SIIN, SIIQFEKL (SIIQ) or SIITFEKL (SIIT) at the concentration specified in the text (10nM – 10pM). The cells were cultured (5% CO_2 at 37^{o}C) for four hours. After incubation, the cells were washed with 200μl PBS then stained and analysed by flow cytometry.

2.7.4 Retroviral transduction of CD8 T cells

Naive OT-I splenocytes were isolated and activated as described previously, using ConA (5μg/ml), IL-7 (1ng/ml) and IL-12 (5ng/ml). Non-treated P6 tissue culture plates were coated with RetroNectin (TAKARA) (30μg/ml) and incubated overnight (4^{o}C). 24 hours post activation, 1x10^7 OT-I cells were resuspended in 2.5ml of the retroviral supernatant, harvested from transfected Phoenix Eco cells and then supplemented with IL-2 (10ng/ml). The cells were placed on a RetroNectin coated P6 well, centrifuged (1800rpm for 90 minutes at 32^{o}C) and cultured for a further 24 hours. The OT-I cells were then suspended (5x10^5/ml) in T cell media supplemented with IL-2 (10ng/ml). Transduction efficiency was assessed either 24 or 48 hours post-transduction, by flow cytometry.

2.8 *In vivo* experiments

2.8.1 Injections

Injection route	Total/maximum volume
Intra-venous (i.v.)	200μl
Sub-cutaneous (s.c.)	200μl

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2.8.2 Collection of peripheral blood

The mouse-tail was locally anesthetised with lidocaine and mice were warmed (at 37°C for 10 minutes). The mouse was then restrained, and the tip of the tail was cut using a scalpel. Blood (5-50µl) was collected and mixed with 4µl heparin (Wockhardt). Samples were stained with mAbs, and red cells were lysed before assessment by flow cytometry.

2.8.3 Tumour inoculation

EG7 tumour cells were cultured for 24 hours in selection antibiotic-free media before administering to the mice by s.c. injection. Tumour size was monitored three times a week using callipers. Mice were sacrificed when the humane endpoint was reached (i.e. when the sum of the two greatest perpendicular measurements was equal to or above 30mm).

2.8.4 Adoptive cell transfer and mice immunisation

The OT-I splenocytes were activated, transduced and cultured as described in the text. Naïve, activated or transduced CD8 T cells ($1x10^4$-$2x10^6$) were resuspended in PBS and transferred to mice by i.v.

For the ACT protocol featuring sublethal irradiation, the mice were irradiated (5.5Gy) using a Gulmay Medical D3225 X-ray)**.** 24 hours later, the mice were inoculated EG7 tumour cells ($2.5x10^5$) by s.c. injection. Five days post tumour challenge the mice were treated with OT-I cells expanded in IL-2. For the ACT protocol not using sublethal irradiation, the mice were inoculated EG7 tumour cells ($2.5x10^5$) by s.c. injection, which was allowed to establish for 1-5 days before administering ACT with IL-2 expanded OT-I cells.

Vaccination consisted of SIINFEKL (30nM) alone or in combination with αCD40 (50-100µg), which were prepared in PBS and injected i.v. into the mice.

2.9 Statistics

GraphPad Prism (V 6.6) software was used to generate graphs and perform statistical analysis of data. All statistics were calculated using a two-tailed unpaired student T-tests unless otherwise stated. Survival curves were assessed with log-rank Mantel-Cox tests to calculate significance. For dose-response curves and tumour growth curves two-way ANOVA with a Bonferroni post-test was performed to calculate significance. Where indicated ns= not significant, * = P<0.05, **=P<0.01, ***= P<0.001, ****=P<0.0001 and error bars show mean +/- SEM unless otherwise stated. When

a statistic is given above data points they are relative to the control group unless otherwise stated.

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Chapter 3: Model optimisation and vector construction

3.1 Introduction

CD8 T cells are specialised lymphocytes that can recognise TAA presented by MHC class I molecules and destroy cancerous cells. As such, selective pressure is put onto tumours to evade and suppress the immune response until the escape phase of immunoediting is entered. Over the past decades, the utility of immunotherapy for the treatment of cancer has been realised. ACT therapy, in which patients are treated with a large number of *ex vivo* expanded tumour-specific lymphocytes, has shown particular success for the treatment of advanced stage melanoma and lymphoma where complete and durable long-term remissions can be achieved [274, 279, 354]. However, not all patients benefit from this therapy and many types of cancers are yet to be successfully treated with ACT [355]. There has been a great deal of interest in genetically augmenting the specificity and function of T cells to improve anti-tumour immunity, but many potential modifications have not been rigorously investigated [274-276, 356, 357]. To explore this area of research, I used a retroviral transduction system to genetically modify OT-I CD8 T cells in preclinically relevant models for ACT.

3.1.1 Retroviral transduction

Retroviruses represent a powerful tool for transducing cells by stably integrating genes into the host genome [358]. Firstly, this process involves the retroviral glycoprotein envelope interacting with surface receptors to enter a cell. Once in the cytoplasm, the retrovirus uses its reverse transcriptase enzyme to produce DNA from positive-sense single-stranded (ss)RNA. During cellular mitosis, the retroviral DNA is incorporated into the host genome using an integrase enzyme, allowing these genes to be stably expressed and inherited by daughter cells. Under normal circumstances, retroviral ssRNA would contain gag, pol, and env genes, which encode catalytic and structural proteins for the construction of new viruses. However, retroviral vectors typically consist of only the proviral sequence; the gene of interest flanked by the long terminal repeats on either side and promoter sequences, such as the human U6 promoter, to enhance its genomic expression [359, 360]. An important factor in the construction of a retrovirus is the use of packaging cell lines, which are commonly derived from the human embryonic kidney line HEK293 due to the high transfection rates achievable from using these cells. As the packaging cell lines express the viral products, gag, pol, and the envelope vector, the introduction of retroviral vector will lead to the production of virions. These retroviral virions are able to transduce cells but lack the ability to replicate outside of the retroviral packaging cell line. Additional, safety

measures can be taken with the use of an ecotropic envelope for the packaging of virions, ensuring that the virus is only able to gain entry into murine cells [359].

The use of retroviral vectors, as well as lentiviral vectors, are relevant to clinical improvements in ACT [299, 357]. As well as the recent success of redirecting CD8 T cells with transgenic TCRs and CARs, augmentation of T cell function and survival is a growing area of research. Within this study, I used the pMKO.1 retroviral vector to generate T cells with targeted protein knockdowns. The pMKO.1 vector contains an MCS specifically intended for the incorporation of a short shRNA oligonucleotide sequence under control of a hU6 promoter. The U6 is bound by an RNA polymerases III enzymes that do not require 5' caps or 3' polyA and is therefore an optimal choice for the precise expression of small mini-genes such as shRNA sequences [361]. Versions of the pMKO.1 vector are currently available with a GFP or a puromycin resistance reporter gene. For many experiments, such as assessing the effect of genetically modifying CD8 T cells on the efficacy of ACT therapy, purified transduced population of cells needed to be generated. By using the pMKO.1 GFP vector, the transduced could be purified by FACS. However, this process can be costly, time-consuming, and requires specialised facilities. Therefore, it was of great interest to modify the pMKO.1 vector to allow compatibility with immunomagnetic cell isolation.

3.1.2 Project aims

Firstly, I aimed to optimise pre-clinical ACT protocols for challenging mice with EG7 tumour cells prior to the adoptive transfer of genetically modified OT-I CD8 T cells. These included protocols for sublethal irradiation preconditioning and post-transfer vaccination, in which the CD8 T cell dose and timing could be optimised to find a window to test the impact of various genetic modification.

The next objective was to modify a pMKO.1 vector to include a reporter gene that would allow for MACS purification of transduced cells. I selected the surface marker CD90.1 (Thy1.1) as it is absent from the Thy1.2 $^{+/+}$ OT-I cells that were to be transduced and WT C57BL/6 mice, which were used as ACT recipients. Provided adequate levels of transduction could be achieved, this vector could then be used for the construction of shRNA vectors.

Finally, work here aimed to create and validate the required retroviral plasmids to conduct my investigations. These assets included the constitutively active (CA) FOXO1, CA FOXO3a, and Eomes genes within the pMP71 vector. Additionally, shRNA sequences targeting PTPN2, PTPN7, PTPN22, and CSK were to be cloned onto the pMKO.1 backbone.

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3.2 Results

As my project aimed to evaluate genetically modified CD8 T cells, I required effective methods for transducing, purifying, and assessing the function of OT-I cells.

3.2.1 Optimisation of the retroviral transduction protocol

During my research I made incremental modifications to optimise the efficiency of CD8 T cell retroviral transduction. Consistent with a previously published transduction protocol [362], activating CD8 T cells with ConA and IL-7, the data here confirm that transduction of T cells activated for 24 hours was more efficient when compared to cells activated for 48 hours (**Figure 3.1A**). Additionally, co-transfection of Phoenix Eco cells with a retroviral packaging vector pCLEco, encoding for gag and pol genes, resulted in a 10-fold enhancement of transduction efficiency (**Figure 3.1A**). The inflammatory cytokine IL-12 enhances CD8 T cell activation, in part due to the upregulation of CD25, and is known to improve engraftment of *in vitro* activated cells upon adoptive transfer [363]. The addition of IL-12 alongside ConA and IL-7 for the activation of CD8 T cells further improved transduction efficiency (**Figure 3.1B**). As IL-12 causes the upregulation of CD25, IL-2 was added alongside the retrovirus to improve transduction efficiency. The maximal transduction rate was observed after 48 hours, although cells could be sorted 24 hours post-transduction with low efficiency. The final transduction protocol consisted of activating naive OT-I splenocytes for 24 hours with ConA, IL-7, and IL-12, transducing cells alongside IL-2, FACS sorting (GFP^+) 24 to 48 hours later and then expanding cells in IL-2 (10ng/ml) to generate CTLs (**Figure 3.1C**).

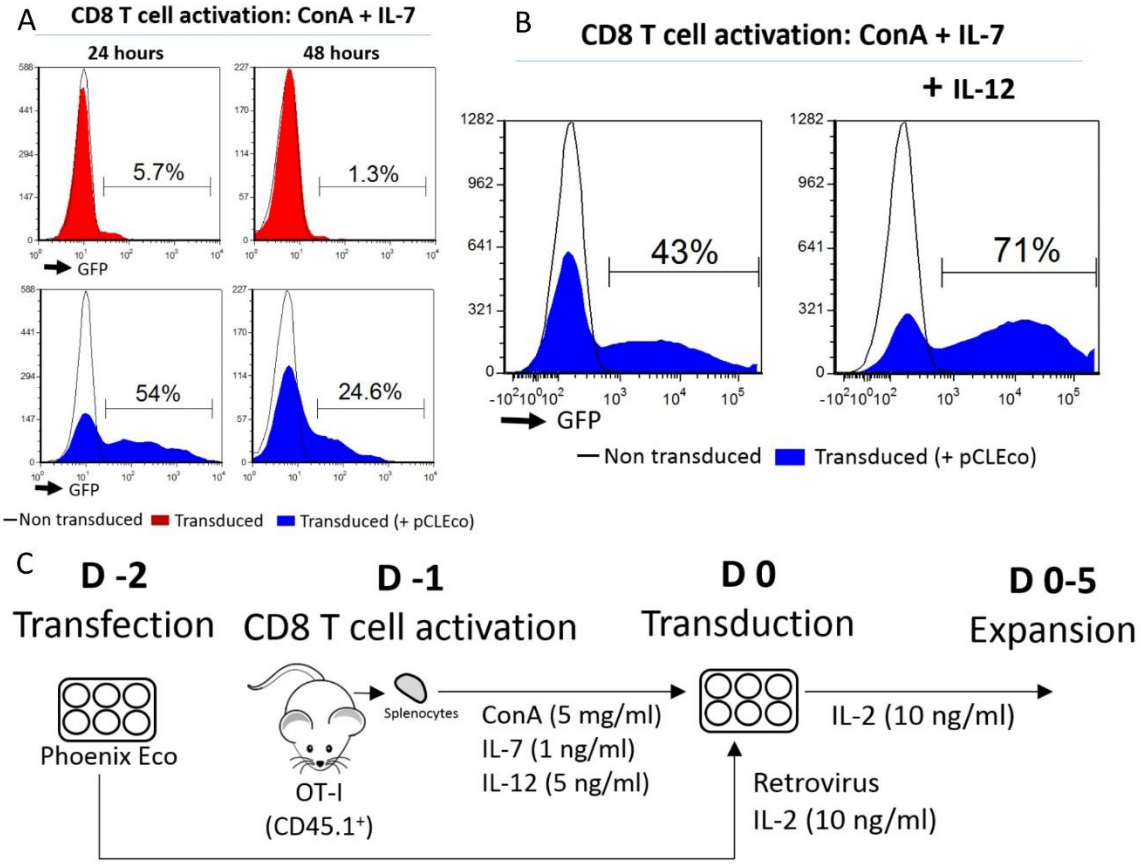

Figure 3.1: Optimising the retroviral transduction protocol. A, Retrovirus was produced by transfecting Phoenix Eco cells with 4μg of the pMKO.1 GFP vector with or without 4μg of pCLEco. Naive OT-I splenocytes were transduced with the pMKO.1 GFP retrovirus, produced with or without pCLEco, at 24 hours or 48 hours after activation with ConA (5mg/ml) and IL-7 (1ng/ml). Transduction efficiency was measured 48 hours post transduction, assessing GFP expression on CD8 T cells by flow cytometry. B, Naive OT-I splenocytes were transduced with the pMKO.1 GFP retrovirus 24 hours after activation with ConA (5mg/ml) and IL-7 (1ng/ml) with or without IL-12 (5ng/ml). The transduction efficiency was measured 48 hours post transduction, assessing GFP expression on CD8 T cells by flow cytometry. C, Schematic protocol detailing the transduction of CD8 T cells to be used in ACT. Briefly, naive OT-I splenocytes ($CD45.1^+$) were activated and transduced with a pMKO.1 GFP retroviral vector. The CD8 T cells were then expanded for five days in IL-2 (10ng/ml) to produce CTLs.

With the optimised transduction protocol established, I then progressed to develop ACT models where transduced CD8 T cells could achieve a moderate level of therapy. In several pre-clinical murine models, mice are preconditioned with sub-lethal irradiation followed by a subcutaneous administration of tumour cells, which are allowed to establish before ACT treatment and subsequent rounds of vaccination are applied [47, 362, 364]. For this work, I used EG7 lymphoma cells. The expression of OVA by this cell line was confirmed by presence of SIIN/H-$2K^b$ complex on

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the cell surface (**Figure 3.2A**). OT-I T cells possess a TCR with a high affinity towards SIIN/H2K^b and could therefore be used to test the function of tumour specific T cells against EG7 tumours. To evaluate the function of OT-I T cells, transduced cells were purified by FACS and expanded for 5 days in IL-2 to generate effector CTL profile [290, 346]. Mice were sublethally irradiated (5.5Gy) and challenged with EG7 tumours, which was then allowed to establish for 5 days prior to ACT therapy. The initial experiment compared high and a low dose of transduced T cells to establish the number of cells required to generate therapy (**Figure 3.2B**). The results showed that no anti-tumour effect was achieved from the transfer of 2.5x10^4 CTLs in this model (**Figure 3.2C and D**). However, therapy with 2.5x10^5 CTLs produced slight but significant improvements in controlling tumour growth and survival duration compared to the untreated controls mice (**Figure 3.2C and D**).

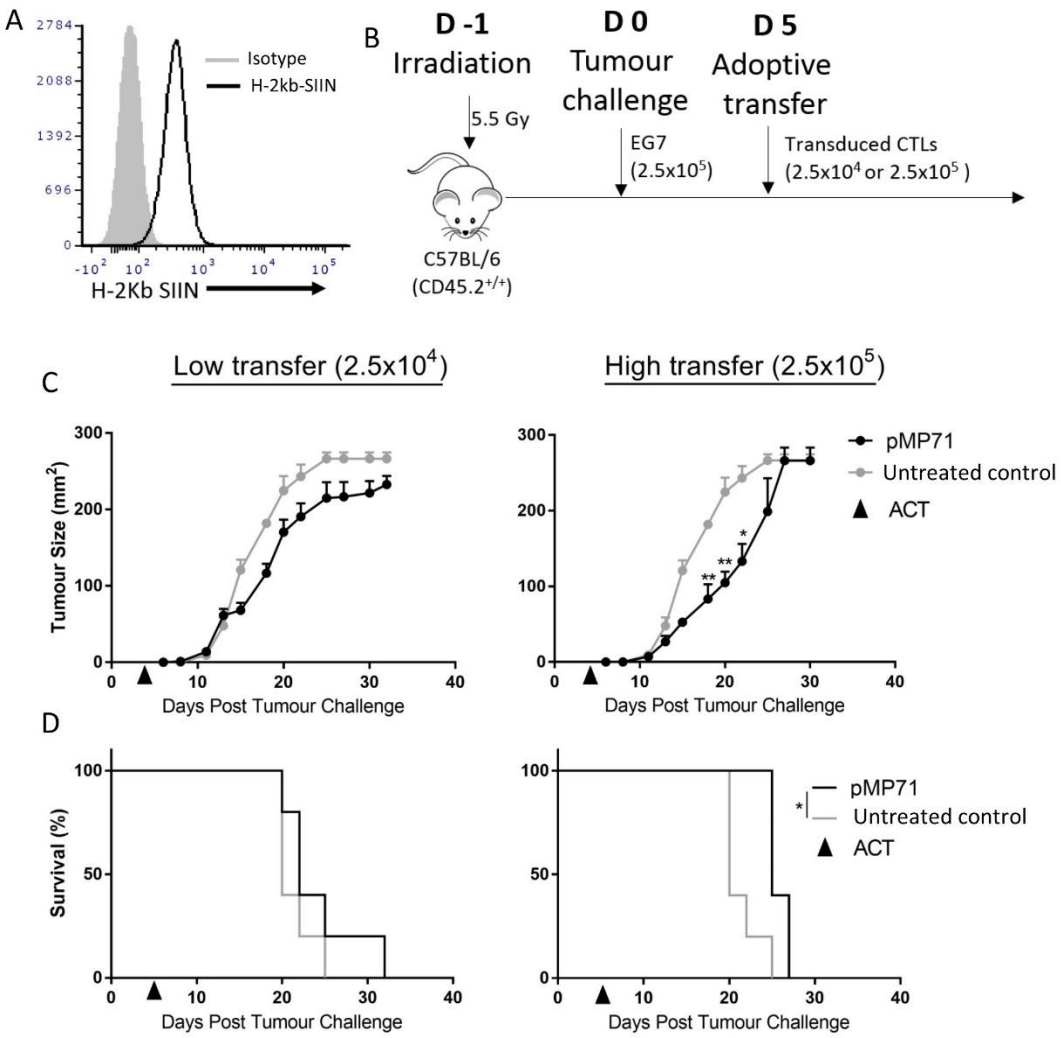

Figure 3.2: The use of OT-I CTLs for ACT therapy against EG7 tumour. A, SIIN/H2K^b expression by EG7 tumour cells was assessed by flow cytometry. B, Schematic detailing the ACT protocol. Briefly, naive C57BL/6 mice were sublethally irradiated (5.5 Gy) 24 hours prior to the subcutaneous administration of EG7 tumour cells (2.5x10^5). Five days post tumour challenge, 2.5x10^4 or 2.5x10^5 CD8 T cells, transduced with pMP71 retrovirus, were adoptively transferred to the mice (indicated by the arrow on the x-axis). C, Tumour size was measured over time, ACT with 2.5x10^4 T cells are shown on the left panel and the 2.5x10^5 transfer is shown on the right. D, Survival was tracked until the terminal end of the experiment, ACT with 2.5x10^4 T cells are shown on the left panel and the 2.5x10^5 transfer is shown on the right. This experiment was conducted once with 5 mice per group.

Experiments were then conducted to establish what extent the OT-I ACT therapy with 2.5x10^5 CTLs could be augmented with the addition of a vaccine. The vaccination consisted of SIIN peptide alone or with a costimulatory αCD40 mAb one-day post adoptive transfer (**Figure 3.3A**). Consistent with previous experiments, the ACT therapy alone produced a small but consistent delay in tumour growth (**Figure 3.3B and C**). The delay in tumour growth achieved by ACT was

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significantly enhanced by the addition of the peptide vaccination. This delay in tumour growth and the survival duration of the mice were significantly enhanced when αCD40 was alongside the peptide vaccination (**Figure 3.3B-D**). This hierarchy in tumour growth control was mirrored by the survival durations of the mice (**Figure 3.3D**). Improved anti-tumour immunity correlated with higher frequencies of transferred cells in the peripheral blood and the proportion of cells expressing KLRG1, a phenotypic marker of effector differentiation. For the ACT only treatment, the transferred OT-I CD8 T cells were detectable at a very low frequency, never reaching more than 0.5% of the total CD8 T cell population nor expressing detectable levels of KLRG1 (**Figure 3.3E**). In contrast, the weak vaccination strategy significantly boosted the frequencies of transferred cells, comprising 35% of the CD8 T cells at day six post transfer, while also resulting in a significant upregulation of KLRG1 by day 14 post transfer. The addition of αCD40 to the peptide vaccination significantly improved the expansion of transferred cells, reaching 85% of CD8 T cells at day 6 post transfer, and again resulted in a significant upregulation of KLRG1 by day 14 post transfer. Taken together, these models generated different levels of ACT therapy, demonstrating an improvement from increasing the strength of the vaccination. These data provided a framework on which to base future experiments designed to evaluate the efficacy of engineered T cells.

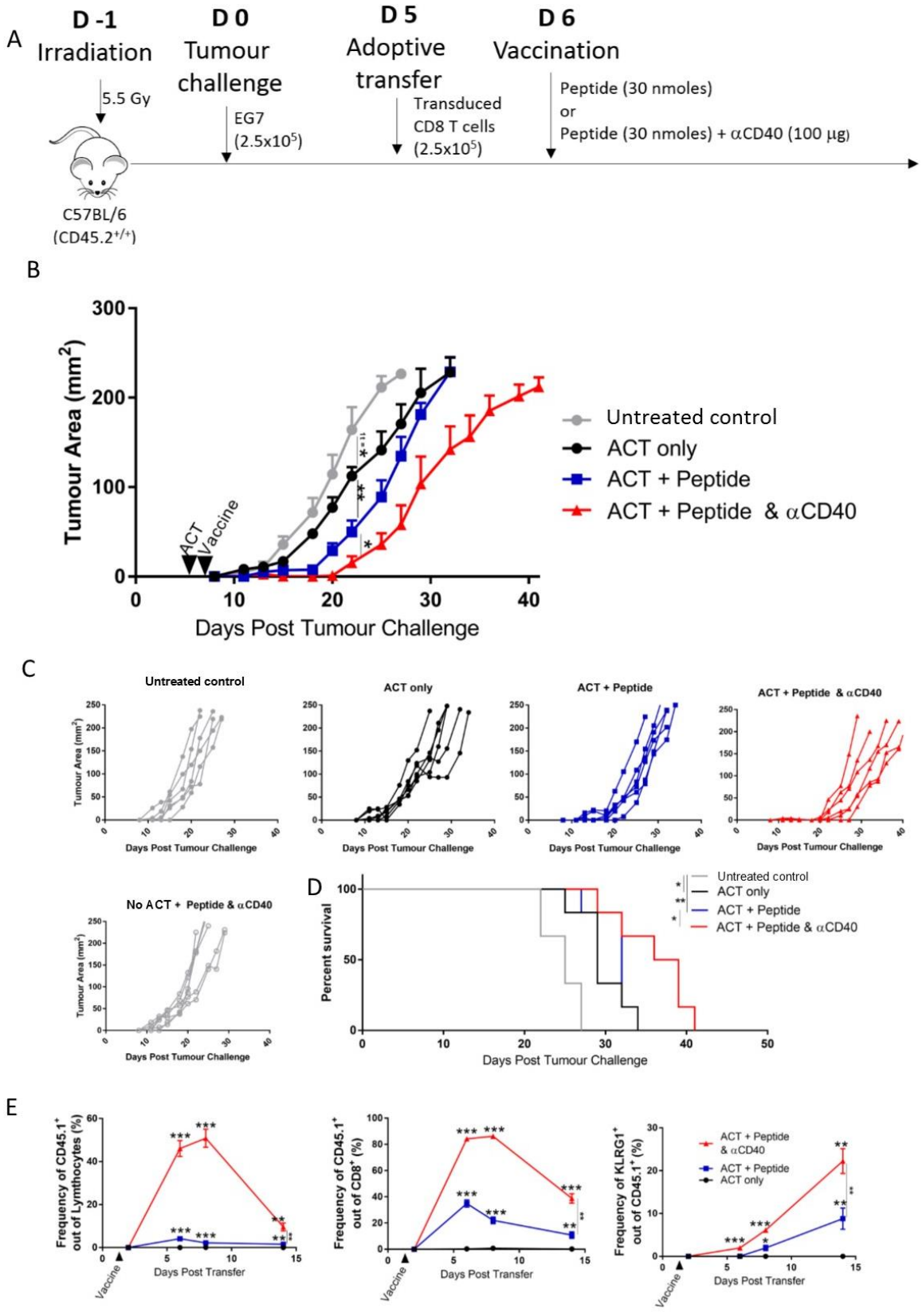

Figure 3.3: Assessing vaccination within the ACT protocol. A, Schematic detailing the ACT model for therapy against EG7 tumours. Briefly, naive C57BL/6 mice were sublethally irradiated (5.5 Gy) 24 hours prior to the subcutaneous administration of EG7 tumour cells ($2.5x10^5$). Five days post tumour challenge, CD8 T cells ($2.5x10^5$) were adoptively transferred into the mice. One-day post transfer, mice were vaccinated with peptide (30nM SIIN) with or without αCD40

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(100µg). B, Average tumour growth is shown over time. C, Tumour growth of individual mice. D, Survival was measured until the terminal endpoint of the experiment. E, Peripheral blood samples were taken at various time points to assess the frequency and phenotype of transferred CD45.1^{+} cells by flow cytometry. All statistics shown here are relative to the ACT only group unless otherwise indicated by bars. The experiment was performed once with six mice per group. The control group consisted of an untreated control (no ACT treatment) and a control group receiving the peptide and αCD40 treatment without ACT.

Finally, it was important to establish an ACT model that was not reliant on irradiating mice prior to ACT. As this presented a suboptimal environment for T cell engraftment, the number of CTLs used for therapy was increased substantially. Following the standard protocol for generating transduced OT-I CD8 T cells, 1x10^{6} cells were transferred to mice bearing five-day established EG7 tumours. In terms of delaying tumour growth or survival benefit, no anti-tumour therapy was achieved (**Figure 3.4A**). Next, the transfer of OT-I cells against five-day established EG7 cells was then attempted alongside the use of a peptide vaccination on the following day. This vaccination protocol achieved a modest, yet significant, improvement in the control of tumour growth but did not impact the overall survival duration of the mice (**Figure 3.4B**). Finally, to create a model which did not rely on vaccination strategies, the adoptive transfer of 1x10^{6} transduced CTLs was performed in mice bearing one day established EG7 tumours. This resulted in a slight, yet significant, delay in tumour growth while also increasing the survival duration of the mice. Although the anti-tumour immunity achieved with these models only resulted in transient delays in tumour growth, this extended the framework for which the genetic modification of CD8 T cells could be assessed in a challenging ACT therapy setting.

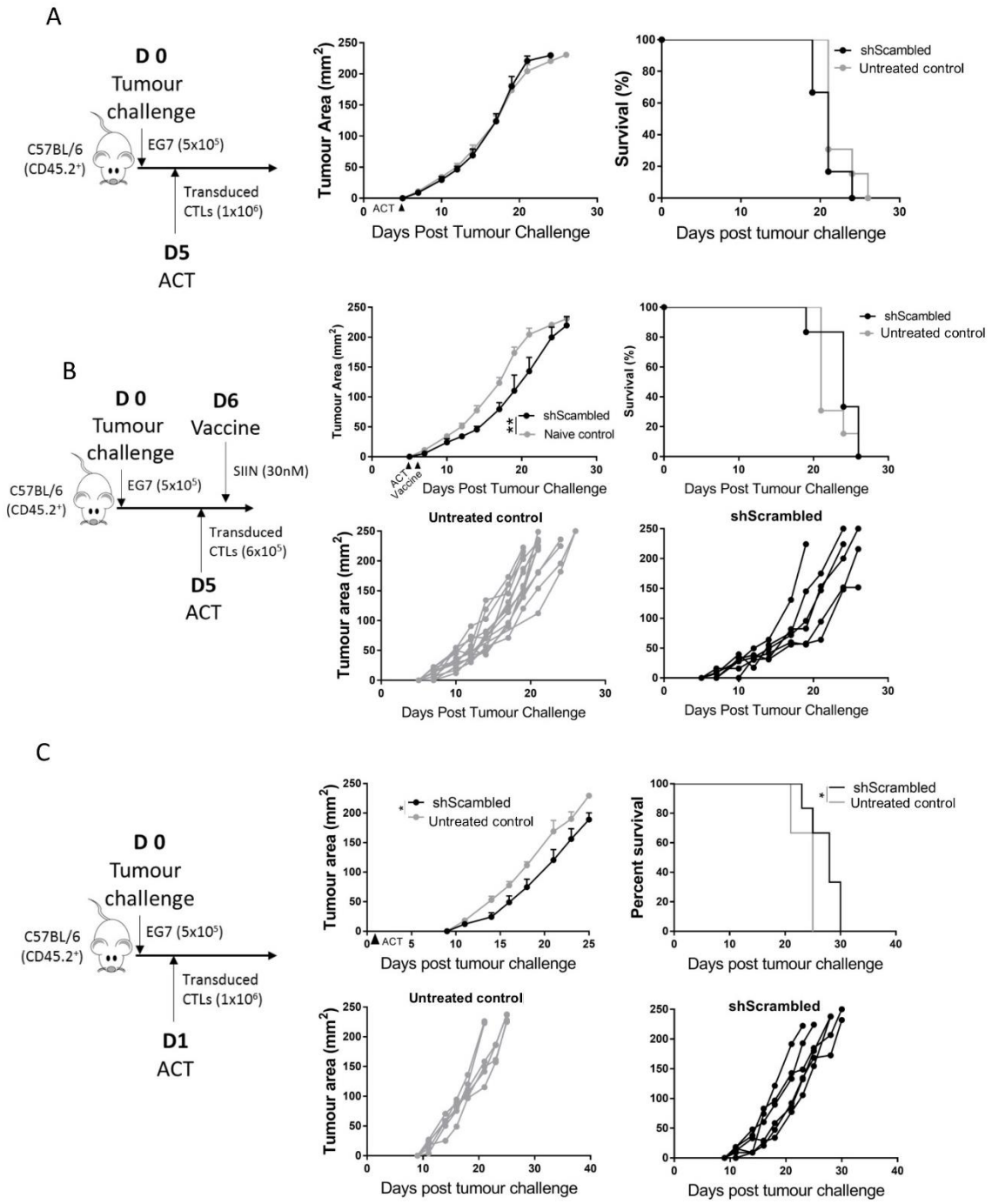

Figure 3.4: ACT in non-irradiated models. Naïve OT-I splenocytes were activated and transduced with shScrambled (control vector) retrovirus following the standard protocol. The transduced CD8 T cells were purified by FACS (GFP^+ $CD4^-$) and expanded for five days in IL-2 (10ng/ml) refreshing the cytokine levels daily. A, Left schematic details the transfer of $1x10^6$ cells into WT C57BL/6 mice bearing five day established EG7 tumours (N=6 mice per group). The middle panel details tumour growth, and the right panel shows the survival duration. B, Left schematic details the transfer of $6x10^5$ cells into WT C57BL/6 mice bearing five-day established EG7 tumours, which was followed by a peptide vaccine (SIIN 30nM) one day later (N=6-12 mice per group). The top middle panel details tumour growth, and the top right panel shows the survival duration. The bottom panels show the tumour growth of individual mice

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with the non-treated control on the left and ACT therapy with shScrambled transduced T cells on the right. C, Left schematic details the transfer of $1x10^6$ cells into WT C57BL/6 mice bearing one day established EG7 tumours (N=6 mice per group). The top middle panel details tumour growth, and the top right panel shows the survival duration. The bottom panels show the tumour growth of individual mice with the non-treated control on the left and ACT therapy with shScrambled transduced T cells on the right.

3.2.2 Development of a retroviral shRNA delivery vector expressing Thy1.1

As several projects required the purification and expansion of transduced CD8 T cells, it was of interest to create a retroviral pMKO.1 vector with a surface reporter gene that would allow immunomagnetic selection. The expression of CD90.1 (Thy1.1) reporter gene could enable the rapid purification of transduced cells without the use of FACS sorting, which is costly and requires specialist equipment. With this project, multiple ligations, purifications, and amplifications were produced at each step. Unique colonies or fragment purifications are reflected by figures having replicates for each product and are numerically labelled (e.g. 1-6).

Firstly, the work here aimed to replace the puromycin resistance gene (Puro) in the pMKO.1 Puro vector with a Thy1.1 gene. The Puro within pMKO.1 Puro was bordered by the restriction sites HindIII and ClaI. However, contrary to the map provided on the Addgene website, diagnostic restriction digestion revealed that multiple HindIII sites were present within the pMKO.1 vector (**Supplementary figures 3.1, 3.2, and 3.3**). Therefore, Puro could not be removed with a simple HindIII + ClaI restriction digest. To gain access to this essential HindIII site, a larger section of pMKO.1 Puro, comprising the SV40 early promoter and Puro, was cloned into a surrogate vector, pBluescript-KS (pBS-KS), in which the Puro could be replaced by Thy1.1 before being transferred back to the pMKO.1 backbone (**Figure 3.5**).

To begin this project, pBS-KS was linearised with the restriction enzymes EcoRI and ClaI, unique sites present in the MCS, to produce Linear pBS-KS (2.9kb) (**Figure 3.6A**). Importantly, this also resulted in HindIII being absent from the Linear pBS-KS vector (**Supplementary figure 3.4**). The pMKO.1 Puro vector was also cut with the restriction enzymes EcoRI and ClaI, producing Linear pMKO.1 (5.7 kb) and SV40+Puro (1 kb) (**Figure 3.6B**). The purified products, SV40+Puro and Linear pBS-KS were ligated together to produce pBS-SV40+Puro (3.9 kb), which was confirmed by a diagnostic restriction digest with HindIII and ClaI to produce fragments of 0.7 kb (Puro) and 3.3 kb (pBS-SV40) (**Figure 3.6C**)

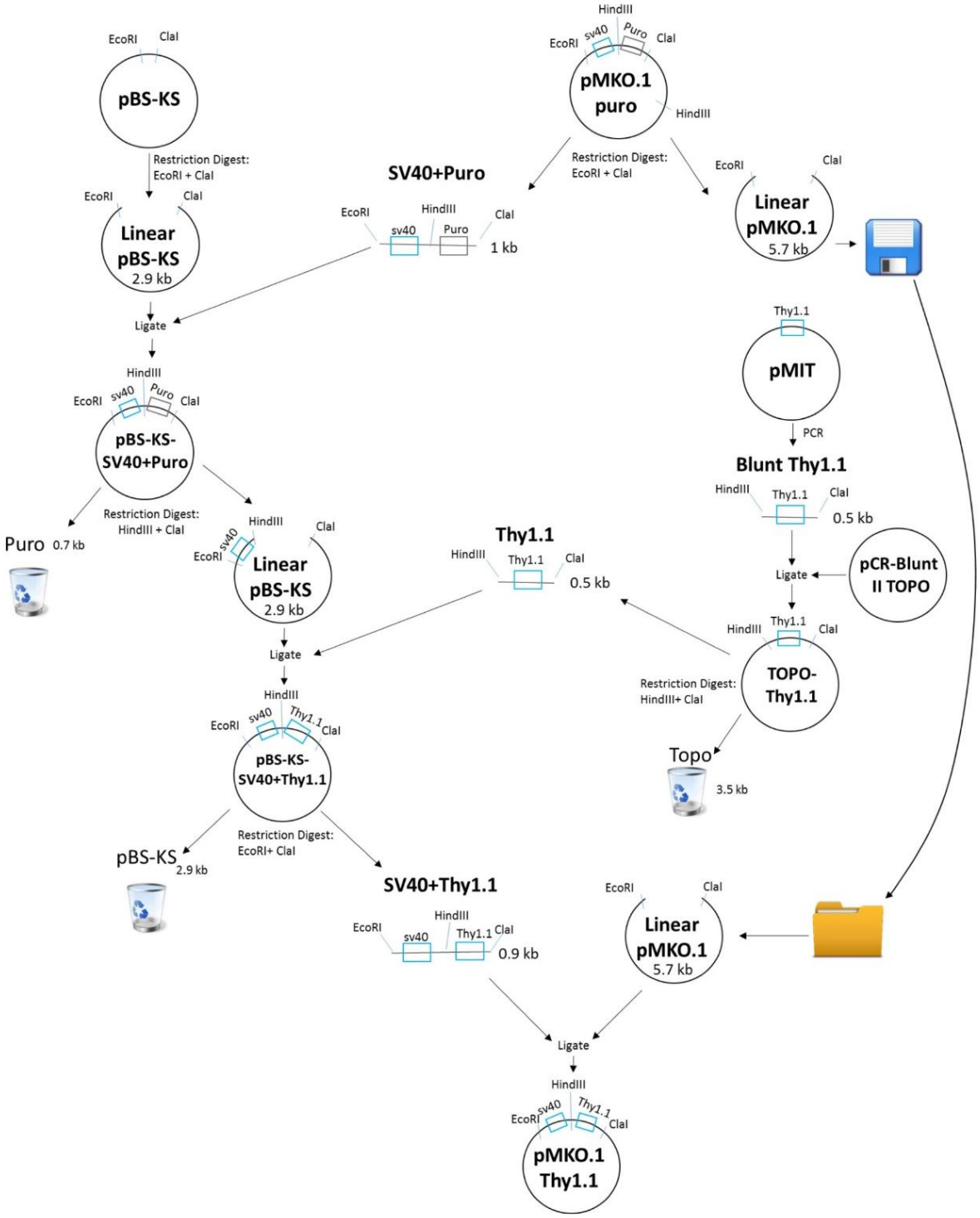

Figure 3.5: A schematic overview of the pMKO.1 Thy1.1 vector construction. Briefly, The SV40 promoter + puromycin resistance region of pMKO.1 Puro was cloned into a surrogate pBluescript-KS vector, from which the puromycin resistance was removed and replaced by Thy1.1 gene that was obtained from a pMiT vector by PCR. The SV40+Thy1.1 was then cloned into the linearised pMKO.1 (lacking the SV40 promoter + puromycin resistance gene), thereby producing the pMKO.1 Thy1.1 vector.

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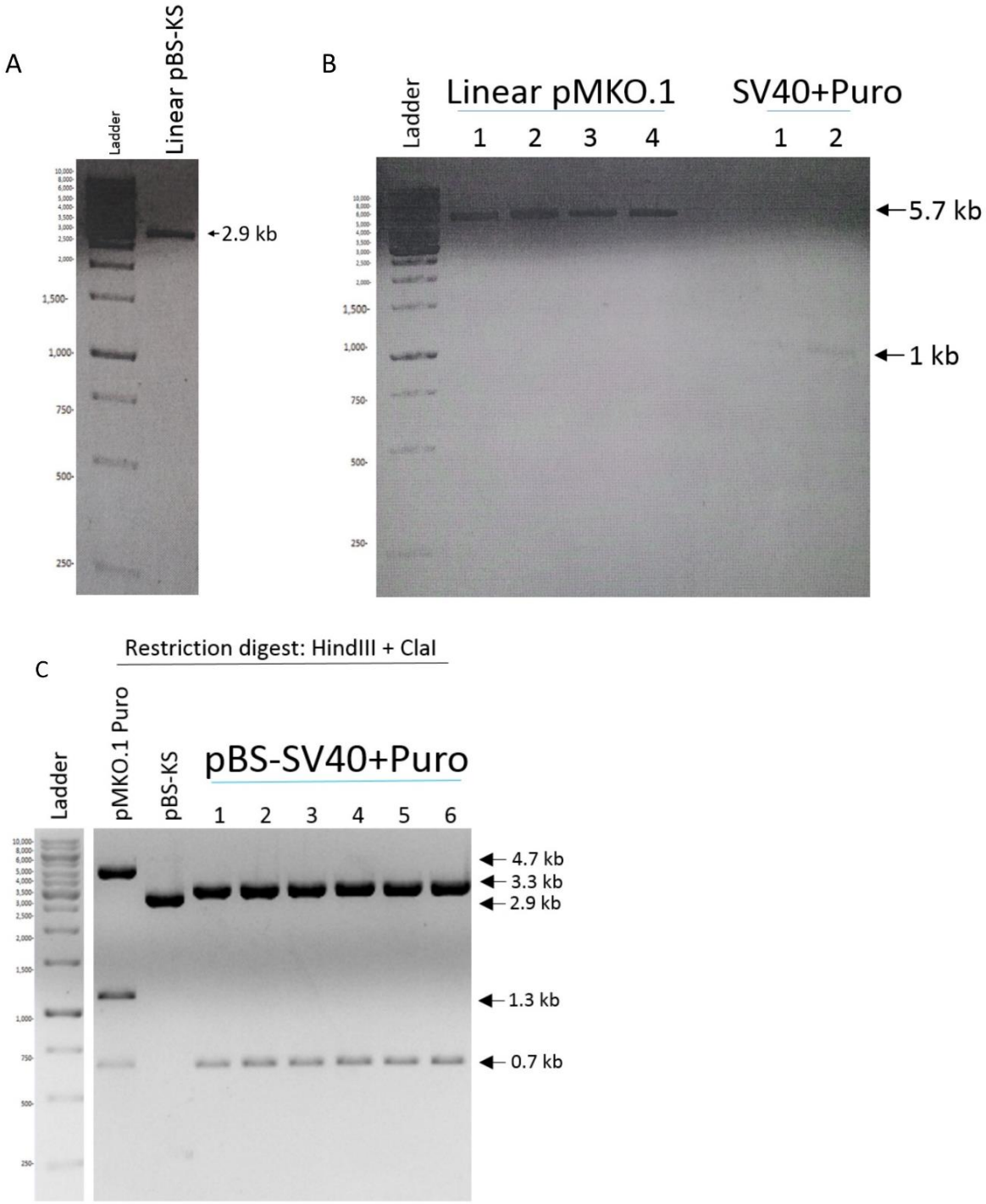

Figure 3.6: Production of pBS-KS-SV40+Puro. A, pBS-KS was cut using the restriction enzymes EcoRI and ClaI to produce Linear pBS-KS (2.9 kb). B, pMKO.1 Puro vector was cut with restriction enzymes EcoRI and ClaI; the gel extraction produced 4 Linear pMKO.1 products (5.7 kb) and two SV40+Puro (1 kb) products. C, The SV40+Puro and Linear pBS-KS products were then ligated together to produce pBS-SV40+Puro vector (3.9 kb), six unique colonies were subject to Miniprep and confirmed by a diagnostic restriction digest with HindIII and ClaI producing fragments of 0.7 kb and 3.3 kb.

As the Thy1.1 gene was less than 600 bp long, it was amplified from the pMIT plasmid by PCR. The primers were designed to extend over Thy1.1 and included overhanging regions featuring the restriction sites required for the subsequent cloning (**Supplementary figures 3.5A and B**). As such, forward primer included a HindIII site and Kozak sequence leading to the Thy1.1 methionine (start codon) site, while the reverse primer included a ClaI site after the Thy1.1 stop codon. To find the optimal PCR conditions two primer sets were designed; in set 1, both forward and reverse primers extended 20 bp into the Thy1.1 gene, which therefore resulted in a shorter reverse primer relative to the forward. For set 2, the reverse primer extension into Thy1.1 was longer while the forward primer was shorter to create equal TM values. Additionally, two PCR programs were used with both sets of primers (**Supplementary figures 3.5C and D**). The results demonstrated that a short primer set with constant annealing temperature provided the optimal amplification condition of Thy1.1 (**Figure 3.7A and Supplementary figure 3.5E**). As a blunt PCR product, the Thy1.1 then ligated into a Blunt-Topo vector, in which the insertion of a DNA fragment disrupts the lethal E. coli gene, ccdB, only permitting the growth of bacteria where positive recombination has occurred [365]. The Thy1.1 insert was confirmed by a diagnostic restriction digest with HindIII and ClaI, producing a 550 bp fragment (**Figure 3.7B**). The Topo-Thy1.1 and pBS-SV40+Puro were digested with HindIII and ClaI to isolate the Thy1.1 and pBS-KS-SV40 respectively (**Figure 3.7C**). Once purified, these products were ligated together to produce a pBS-KS-SV40+Thy1.1 vector (3.9 kb), validated by restriction digests with EcoRI and ClaI to produce fragments of 2.9 kb and 0.95 kb (**Figure 3.7D**). The pBS-KS-SV40+Thy1.1 vector was cut using restriction enzymes EcoR1 and HindIII to produce an SV40-Thy1.1 fragment (0.95 kb) (**Figure 3.8A**). The purified SV40+Thy1.1 fragment possessed the complementary sticky ends, allowing for its ligation with the Linear pMKO.1 fragment produced earlier (shown in **Figure 3.6B**). The completed pMKO.1 Thy1.1 vector construct was confirmed by diagnostic restriction digestion with EcoRI and ClaI to produce 5.7 kb and 0.95 kb fragments (**Figure 3.8B**).

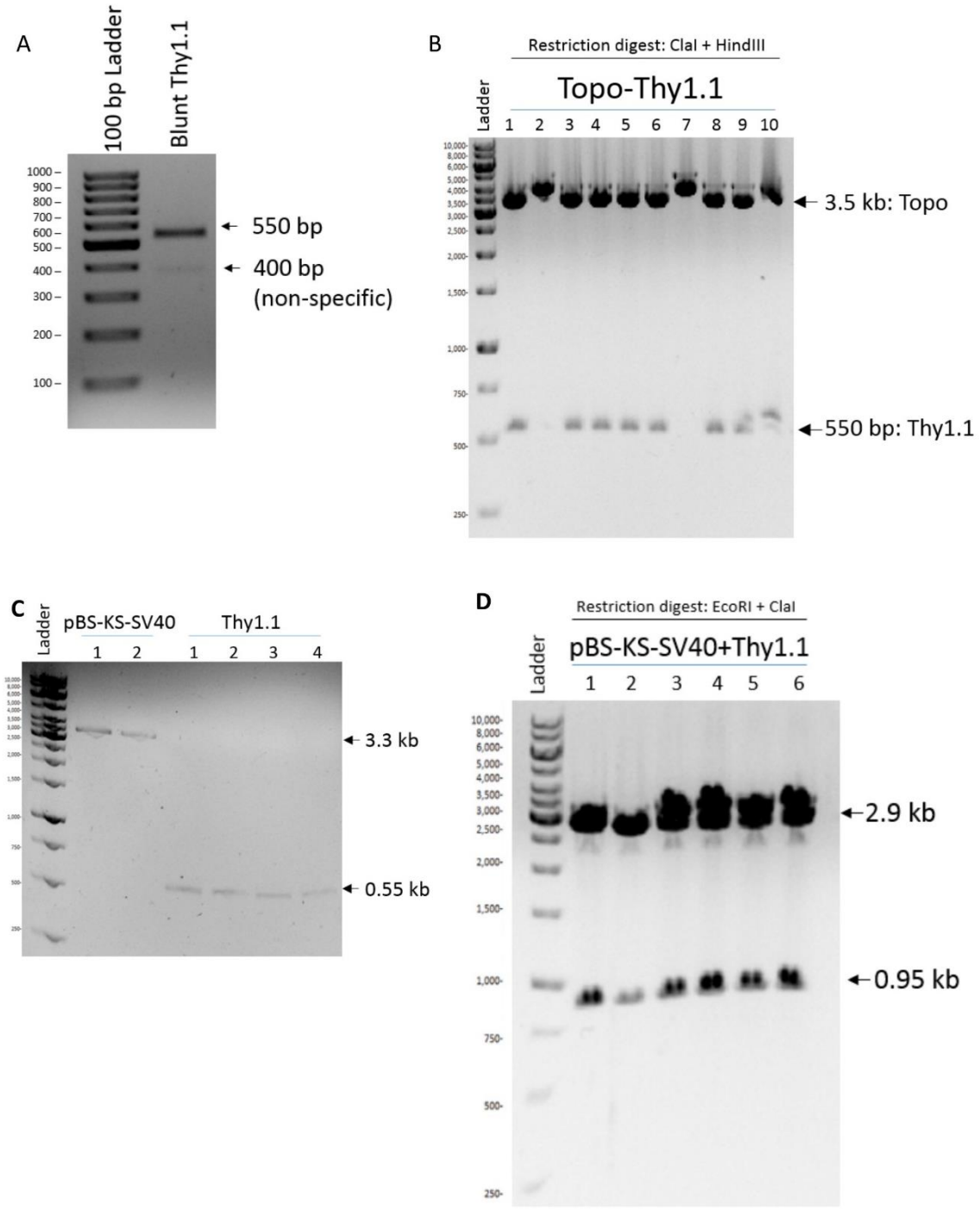

Figure 3.7: Production of pBS-SK-SV40+Thy1.1. A, The Thy1.1 (550 bp) gene was amplified from the pMiT vector by PCR. B, The Thy1.1 fragment was then ligated into the Blunt-Topo vector. From 10 unique colonies Miniprep products were validated by restriction digestion with ClaI and HindIII to produce Thy1.1 (550 bp) and Topo fragments (3.5 kb). C, The pBS-KS-SV40+puro vector was cut with the restriction digestion enzymes HindIII and ClaI to remove the Puro gene producing two products from gel purification. The Topo-Thy1.1 was also digested with HindIII and ClaI to isolate the Thy1.1 gene (0.55 kb) producing four products from gel purification. D, The pBS-KS-SV40 and Thy1.1 fragments were then ligated together produce the pBS-KS-SV40+Thy1.1 vector (3.9 kb). Miniprep products from six unique colonies were then validated by an EcoRI and ClaI restriction digestion to produce the SV40+Thy1.1 fragment (950 bp).

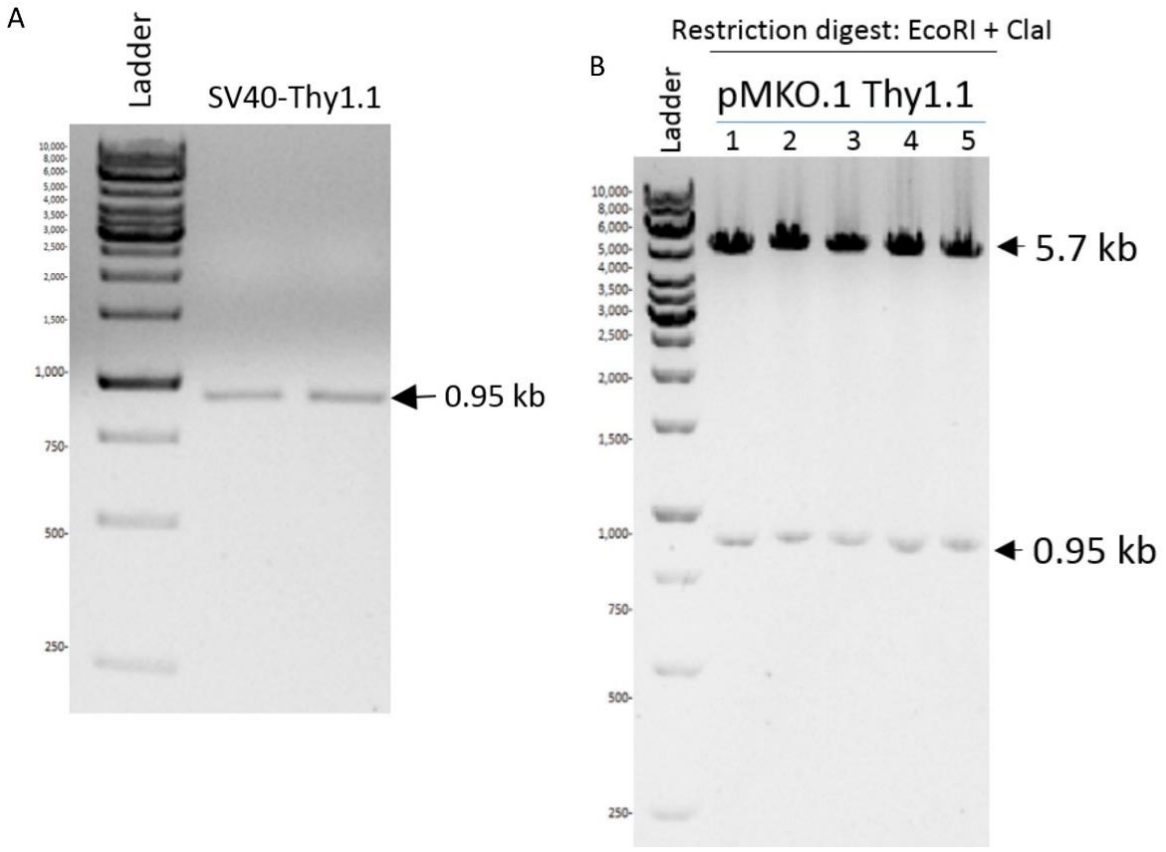

Figure 3.8: Production of pMKO.1 Thy1.1. A, The pBS-KS-SV40+Thy1.1 vector was cut using restriction enzymes EcoRI and HindIII to produce a SV40-Thy1.1 fragment (0.95 kb) producing two products from gel purification. B, The SV40+Thy1.1 was then ligated with Linear pMKO.1 fragment (as shown in **Figure 3.6B**), to produce the pMKO.1 Thy1.1 vector. The Miniprep products of five unique colonies were validated by restriction digestion with EcoRI and ClaI to produce a 0.95 kb and 5.7 kb fragment.

The newly constructed pMKO.1 Thy1.1 vector (**Figure 3.9A**) was of a similar size to the pMKO.1 puro, at approximately 6.7 kb, but could be distinguished through a ClaI and EcoRI restriction digest producing a fragment of 0.95 kb compared to 1 kb (**Figure 3.9B**). To validate the functionality of the pMKO.1 Thy1.1 vector, it was compared to empty control vectors, pMKO.1 GFP and pMIT, within the transfection and transduction protocol described in **Figure 3.1C**. The results showed that the Phoenix Eco retroviral packaging cell line, transfected with pMKO.1 Thy1.1, expressed adequate levels of Thy1.1 (**Figure 3.9C**). However, the retrovirus produced from these transfections failed to transduce T cells (**Figure 3.9D**).

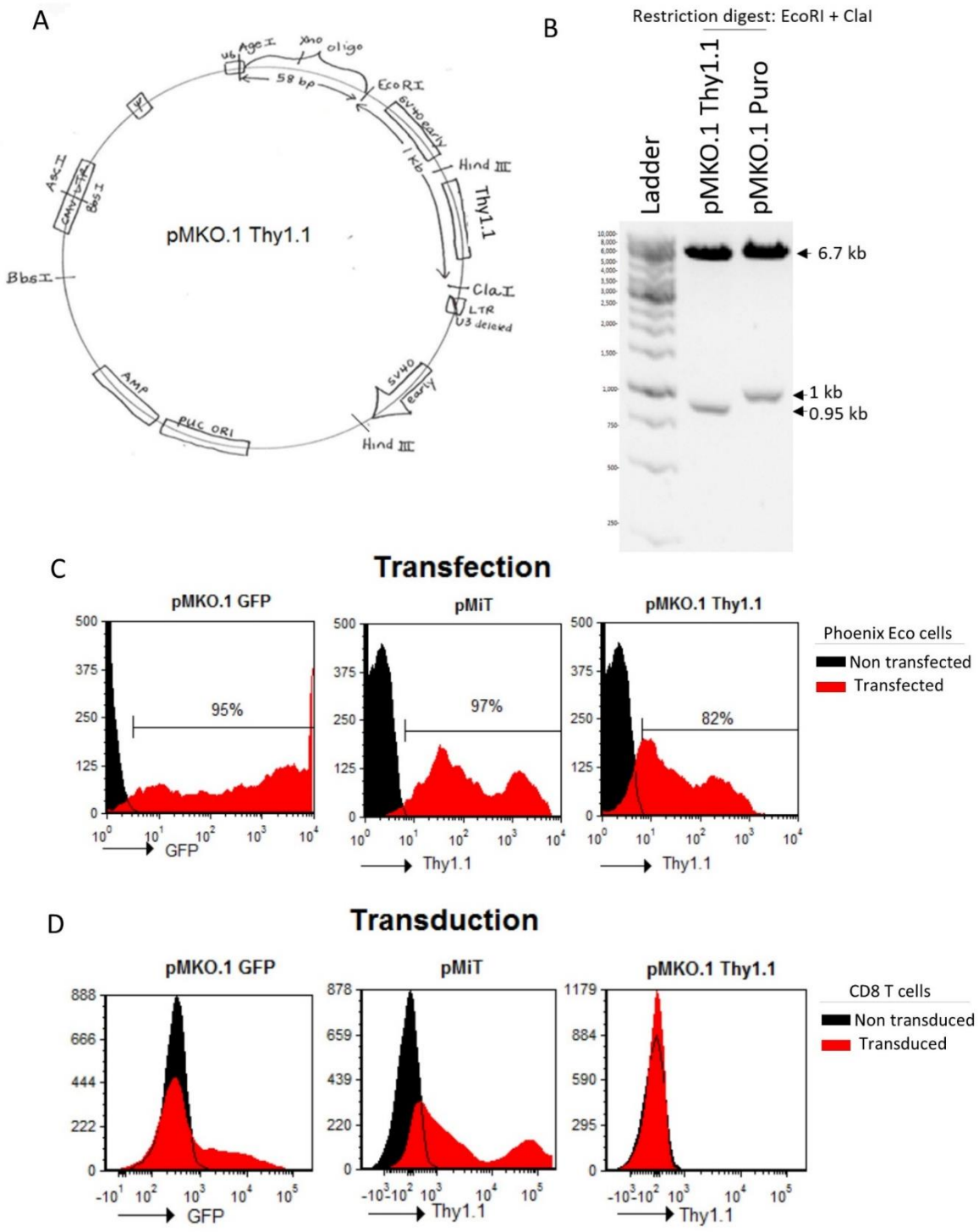

Figure 3.9: Validation of the pMKO.1 Thy1.1 vector. A, Schematic map of the pMKO.1 Thy1 vector. B, The pMKO.1 Thy.1.1 vector and pMKO.1 GFP vector were cut with restriction enzymes (EcoRI and ClaI), producing 0.95 kb and 1 kb fragments respectively. C, Transfection of Phoenix Eco with either the pMKO.1 GFP, pMiT, or pMKO.1 Thy1.1 vector. The GFP or Thy1.1 expression was analysed by flow cytometry 48 hours post-transfection. D, Naive OT-I splenocytes were activated in ConA and IL-7 for 24 hours before transduction with either pMKO.1 GFP, pMiT, or pMKO.1 Thy1.1 retrovirus. The GFP or Thy1.1 expression of CD8 T cells was analysed by flow cytometry 48 hours post-transduction.

The expression of Thy1.1 on the Phoenix Eco cells validated functional SV40 mediated Thy1.1 expression, yet clearly the addtion of this sequence dirusbted the ablilty of of the vecotor to produce functional retrovirus that could transduce cells. It is possible that either a poor viral titer or compromised ability to incorporate LTR flanked genes into the host genome could account for this problem. As the pMKO.1 GFP vector had been validated by our lab it was of interest to investigate if this vector backbone was more suitable for the incorporation of SV40-Thy1.1 to produce a functional retroviral product. This stage of the project involved the removal of the SV40 promoter from the pMKO.1 GFP and insertion of in the SV40-Thy1.1 fragment to produce a pMKO.1 GFP Thy1.1 vector (**Figure 3.10**). After diagnostic assessment of the pMKO.1 GFP vector, it was established that the map provided by Addgene contained several errors, with a ClaI site in the MCS 5' of the GFP, while the ClaI site 3' of the GFP was no longer present (**Supplementary figure 3.1 and 3.2**). However, this situation allowed for a restriction digest with ClaI and EcoRI to remove the SV40 effectively without disrupting the GFP gene, producing a pMKO.1 GFP Δ SV40 fragment. The SV40-Thy1.1 fragment produced earlier (**Figure 3.7A**) therefore possessed complementary sticky ends, with EcoRI and ClaI, to be ligated into pMKO.1 GFP Δ SV40, producing the pMKO.1 GFP Thy1.1 vector (**Figure 3.11A**). This construct was validated by restriction digest with EcoRI and ClaI, producing a 0.95 kb fragment (**Figure 3.11B**). The pMKO.1 GFP Thy1.1 vector was then used in the standard transduction protocol and compared to the empty retroviral vectors pMKO.1 GFP and pMiT. The results demonstrated that the pMKO.1 GFP Thy1.1 vector could successfully transfect Phoenix Eco cells, resulting in co-expression of GFP and Thy1.1 (**Figure 3.11C**). Importantly, the retrovirus produced in this experiment was also successful in transducing CD8 T cells, as shown by the GFP^+ $Thy1.1^+$ population present (**Figure 3.11D**). However, it was apparent that the transduction efficiency was limited by the addition of Thy1.1 into the pMKO.1 GFP vector, showing a three-fold reduction in the frequency of transduced cells compared to the control group (**Figure 3.11D**). Therefore, despite the advantages afforded by a congenic surface reporter gene, the low transduction efficiency made this vector a poor replacement for the pMKO.1 GFP, which could yield much higher numbers of transduced cells. As such, the pMKO.1 GFP vector was used for the subsequent cloning of shRNA retroviral vectors. However, future work could focus on removing the redundant GFP gene from pMKO.1 GFP Thy1.1, with a restriction digest of ClaI and ECORV or NheI, and explore whether this would restore the transduction efficiency to an appropriate level and validate the utility of this vector.

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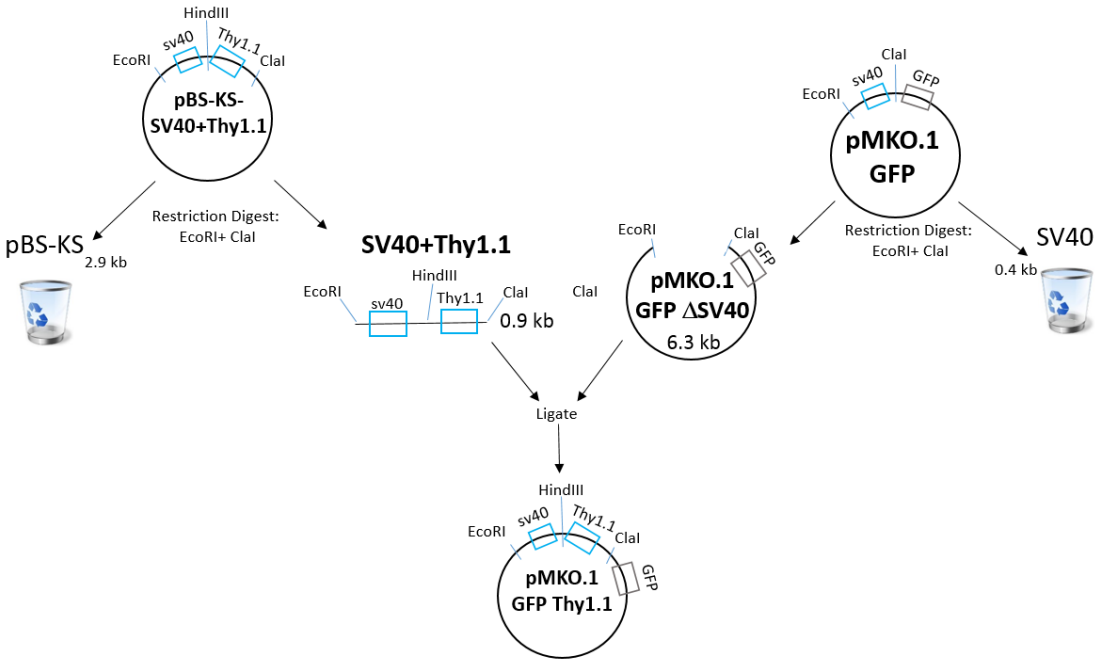

Figure 3.10: A schematic overview of the pMKO.1 GFP Thy1.1 vector construction. Briefly, the pMKO.1 GFP vector was cut with the restriction enzymes EcoRI and ClaI to produce a linearised pMKO.1 GFP ΔSV40 fragment. This pMKO.1 GFP ΔSV40 fragment was ligated with the SV40+Thy1.1 fragment (produced in **Figure 3.8A**) to produce the pMKO.1 GFP Thy1.1 vector.

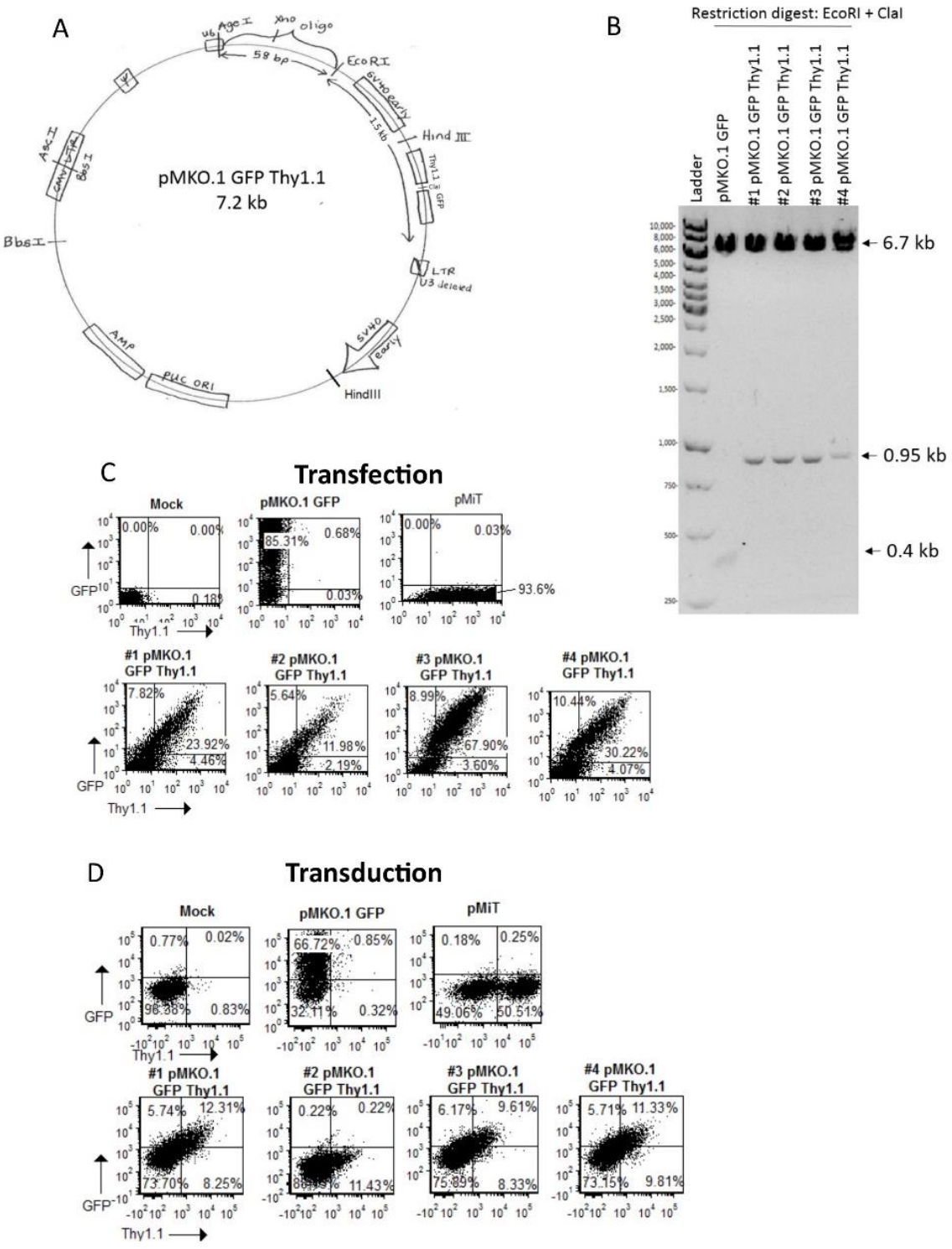

Figure 3.11: Validation of pMKO.1 GFP Thy1.1 vector. A, Schematic map detailing the pMKO.1 GFP Thy1.1 vector. B, Four unique colonies Maxiprep products for the pMKO.1 GFP Thy1.1 vector and a pMKO.1 GFP vector were cut with the restriction enzymes EcoRI and ClaI, producing a 950 bp fragment and 400 bp fragment respectively. C, Transfection of Phoenix Eco with either the pMKO.1 GFP, pMiT, or pMKO.1 GFP Thy1.1 vectors. The GFP and Thy1.1 expression were analysed by flow cytometry 48 hours post-transfection. D, Naive OT-I splenocytes were activated in ConA and IL-7 for 24 hours before transduction with either pMKO.1 GFP, pMiT, or pMKO.1 Thy.1.1 GFP retrovirus. The GFP and Thy1.1 expression of CD8 T cells were analysed by flow cytometry 48 hours post-transduction.

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3.2.3 Construction of shRNA retroviral vectors

For the effective shRNA mediated knockdown genes, 21 base pair sequences were generated using the Broad Institute's public RNAi search tool (http://www.broadinstitute.org/rnai/public/gene/search). This tool ranks shRNA sequences based on Rule Set 2 on-target score and the Cutting Frequency Determination (CFD) score to assess off-target interaction, alongside additional criteria such as cut position and mutual spacing. The top four scoring sequences, which registered no off-target reactivity, were selected for murine PTPN7, PTPN22, and CSK (labelled A-D). The PTPN2 shRNA sequence was obtained from a previously validated shRNA library [113], and a scrambled control sequence was randomly generated with 50% G/C content. The shRNA oligos were synthesised by Life Technologies using their 'Custom DNA Oligo' services. The complementary forward and reverse oligos were annealed together, generating double-stranded fragments with AgeI and EcoRI overhangs (**Figure 3.12 A**). The annealed oligos were then ligated into the pMKO.1 GFP vector, which had been linearised with the restriction enzymes AgeI and EcoRI. The insertion of the shRNA sequence, containing a XhoI site, was validated by a restriction digest with HindIII and XhoI or XhoI alone (**Figure 3.12 A**). These digests produced a 400 bp fragment that was not present for the empty pMKO.1 GFP vector, confirming the presence of shRNA inserts in all constructs (**Figure 3.12 B and C)**.

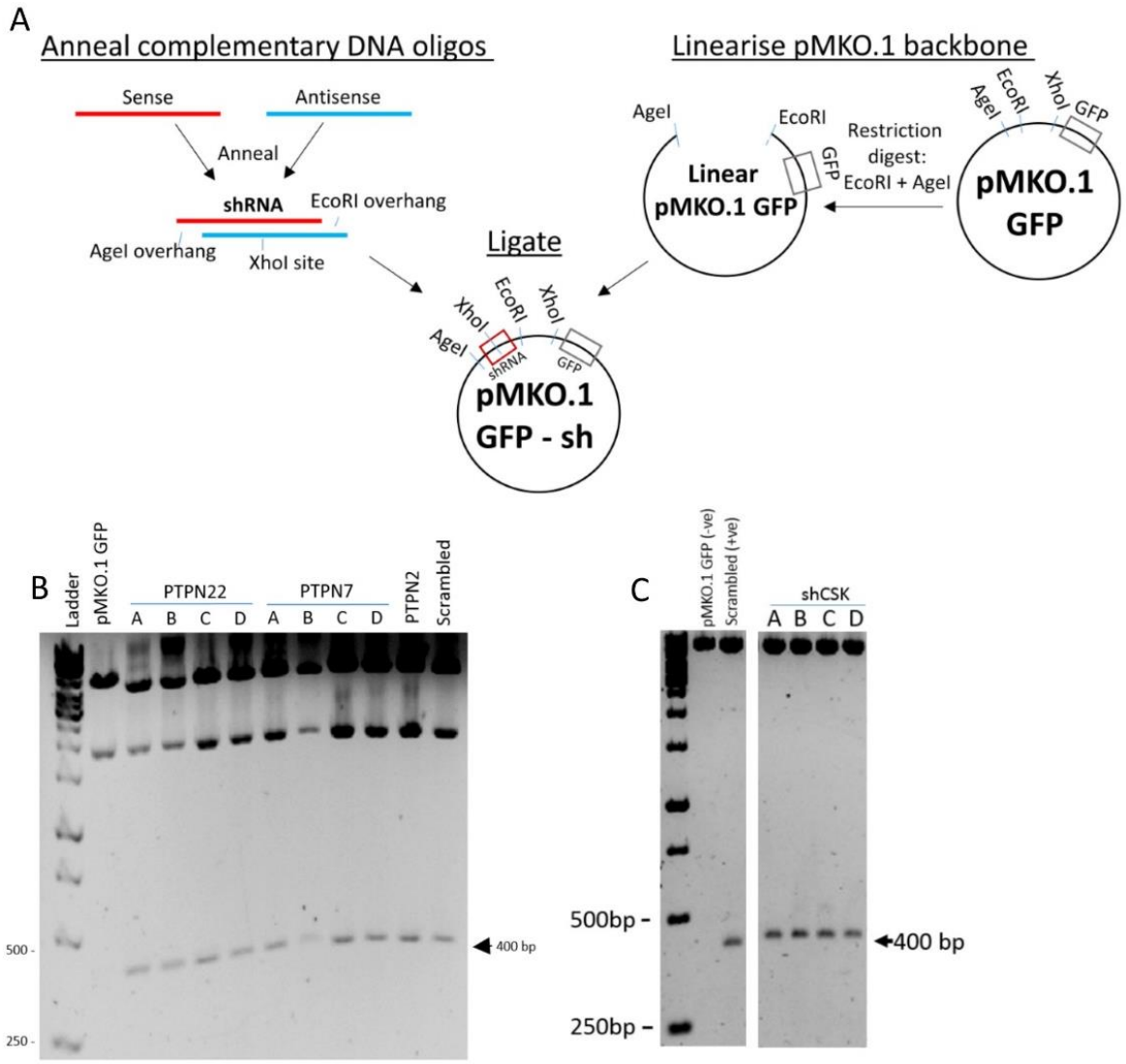

Figure 3.12: Construction of shRNA vectors. A, A schematic detailing the construction process. Briefly, pMKO.1 GFP was linearised using an EcoRI and AgeI restriction digest. The sense and antisense oligos were annealed, creating the complementary overhang sites for AgeI and EcoRI and allowing for the ligation into the linearised pMKO.1 GFP vector. B, The insertion of the shRNA was validated using a diagnostic XhoI and HindIII restriction digest to produce a 400 bp fragment that was not present in the empty vector. C, The insertion of the shRNA was validated using a diagnostic XhoI restriction digest to produce a 400 bp fragment that was not present in the empty vector.

3.2.4 Construction of overexpression vectors

To address the role of transcription factors for CD8 T cell function and differentiation, several vectors were constructed for the overexpression of certain genes. The gene of interest's exon coding sequence was obtained from the NCBI web site. A NotI restriction site and Kozak consensus sequence were then added to the 5' end, and a SalI restriction site was added to the 3' end of the sequence. The constructs were synthesised using the Invitrogen GeneArt Gene Synthesis service. The GeneArt plasmids were digested with NotI and SalI restriction enzymes to

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extract the desired sequence for cloning into an expression vector. For the construction of a plasmid expressing Eomes, the sequence was ligated into the MCS of pMP71, which had been linearised with NotI and SalI restriction enzymes. The presence of the Eomes insert was then validated by a NotI and SalI restriction digest, producing a fragment of 2150bp not present in pMP71 (**Figure 3.13 A**). Following the protocol described in **Chapter 3.2.1**, CD8 T cells transduced with pMP71 or pMP71-Eomes, which were then FACs purified by gating on GFP^+ cells, and expanded for five days in IL-2. The results here demonstrate that the retention of transduced cells post-sorting maintained >99% purity (**Figure 3.13B**).

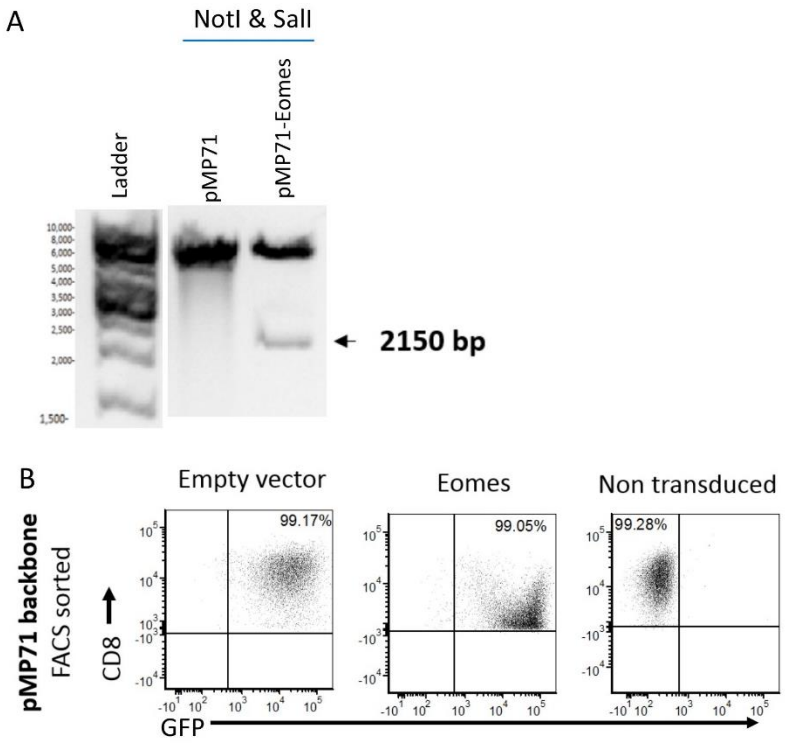

Figure 3.13: Construction and validation of Eomes retroviral vectors. A, Following the ligation of the Eomes fragment into retroviral vectors, pMP71 and pMP71-Eomes were digested with NotI and SalI restriction digestion enzymes, with the band at 2150bp present for the construction containing Eomes assessed by gel electrophoresis. B, Naïve OT-I splenocytes were activated and transduced with pMP71 or pMP71-Eomes retrovirus following the standard protocol. The CD8 T cells transduced with retrovirus on the pMP71 background were purified by FACS (GFP^+ $CD4^-$) five days in IL-2 before assessing the purity of transduced cells by FACS, gated against non-transduced control cells.

Our lab had previously synthesised FOXO1 AAA and FOXO3a AAA genes, with three alanine mutations at the Akt phosphorylation sites, which were cloned into the pMP71 retroviral vector. To confirm the presence of these genes within the vectors, I performed a restriction digest using

HapI and NotI, which produced a 420bp band for FOXO1 AAA and a 1.4kb band for FOXO3a AAA that are not present in the pMP71 control vector (**Figure 3.14A and B**). Next, the ability of the pMP71, FOXO1 AAA, and FOXO3a AAA vectors to transduce T cells was validated following the protocol established. Two days post-transduction typically 10-60% of the CD8 T cells were shown to express GFP, with higher efficiency shown for the pMP71 vector compared to the FOXO1 AAA or FOXO3a AAA (**Figure 3.14C**).

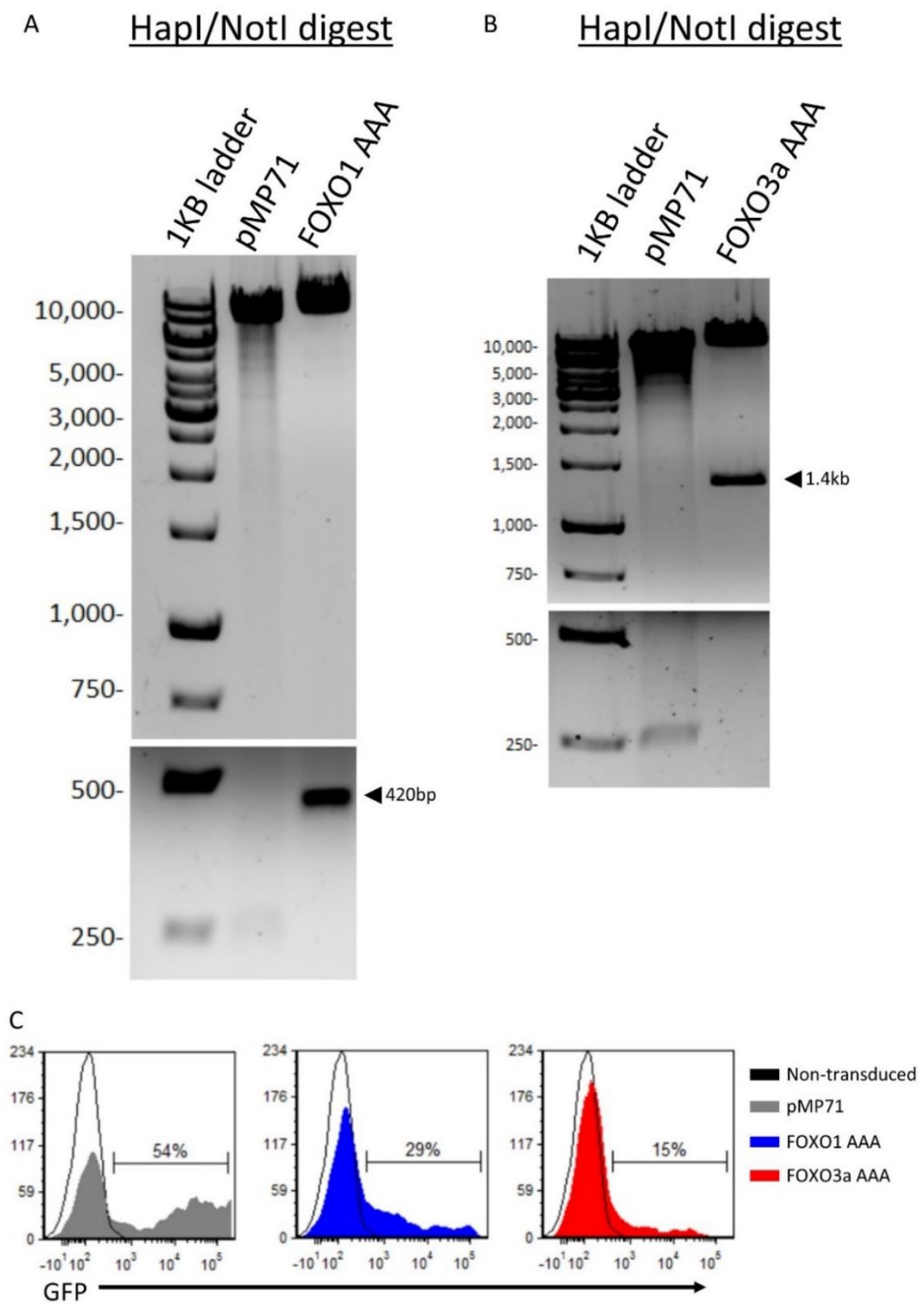

Figure 3.14: Validation of FOXO overexpression vectors. A, The FOXO1 AAA vector was digested using HapI and NotI restriction enzymes, producing a fragment at 420bp not present for the pMP17 control vector. The gap present in the image represents images taken with different exposure times of the same gel. B, The FOXO3a AAA vector was digested using HapI and NotI restriction enzymes, producing a fragment at 1.4kb not present for the pMP71 control vector. The gap present in the image represents images taken with different exposure times of the same gel. C, Naïve OT-I T cells were transduced with FOXO1 AAA, FOXO3a AAA, or pMP71 retrovirus and expanded for two days in IL-2. The transduction efficacy was measured as the proportion of cells expressing GFP, determined by flow cytometry.

3.3 Discussion

Although the adoptive transfer of *ex vivo* expanded CD8 T cells can mediate the regression of certain cancers, ACT efficacy is hindered by low engraftment rates and the inherent immunosuppressive tumour microenvironment limiting T cell stimulation [279, 354]. Recent advances in genetic engineering have broadened the applicability of ACT therapy to treat new cancer types, yet this technology also has the potential to modulate molecular pathways and thereby improve the survival and responsiveness of transferred T cells [274, 276, 279]. As such, there are many untested genetic alterations that warrant investigation to elucidate whether the efficacy of ACT against cancer can be enhanced. Data presented in this chapter focused on optimising a preclinical ACT model using transduced OT-I CD8 T cells in various EG7 tumour settings, alone or in combination with irradiation and/or vaccination. Efforts were also made to broaden the retroviral tools available by cloning a surface Thy1.1 marker into the pMKO.1 vector. Finally, the vectors required to study the genetic manipulation of CD8 T cells were constructed.

Published methods for the retroviral transduction of CD8 T cells are based on the use of ConA and IL-7 to activate CD8 T cells 24 hours prior to transduction [362]. Theoretically, to achieve higher levels of transduction, a greater proportion of cells need to be cycling. Therefore, to enhance transduction efficacy, the inflammatory cytokine IL-12 was added alongside ConA and IL-7 for the activation of CD8 T cells, to mediated CD25 upregulation and thereby sensitise cells to IL-2 mediated proliferation. As such, highest rates of transduction were achieved with the addition of IL-2 alongside retrovirus during the transduction process. With this optimised protocol, certain retroviral vectors were able to achieve the transduction of 75% of CD8 T cells in the population. Shortly after completing this work, the finding that IL-12 was beneficial to retroviral transduction of T cells was corroborated by a study drawing the same conclusions [363]. As well as the improvements gained for transduction efficiency, short exposure to IL-12 during T cell activation *in vitro* can also help improve the engraftment of CD8 T cells post adoptive transfer [366, 367].

To study the anti-tumour activity of genetically modified CD8 T cells for ACT, I required murine models that would allow for the assessment of improved anti-tumour immunity. Previous studies have established that the ACT of pmel CD8 T cells ($1x10^4$-$1x10^5$) alongside vaccinations can generate anti-tumour immunity in the mouse B16 melanoma model [362]. The use of vaccination in ACT is clinically relevant when considering cases where a known TAA is being targeted by TCR-transgenic T cells. To establish an ACT model, I transferred transduced OT-I cells to sub-lethally irradiated mice bearing EG7 tumours, which then received vaccinations. The addition of vaccines

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to ACT significantly enhances the expansion of the transferred CD8 T cells and results in the upregulation of KLRG1, a marker of effector differentiation [45]. These factors accounted for significant delays in tumour growth. Perhaps unsurprisingly, the normal growth rate returned following the contraction of transferred CD8 T cells presenting a correlation between frequency of T cells and tumour control. Additional efforts were made to establish an ACT protocol that was not reliant on sub-lethal irradiation precondition, due to a change in available facilities halfway through my project. This protocol provided a more stringent environment for transferred T cells to mediate anti-tumour immunity, without lymphopenia to aid in engraftment and remove suppressive T_{regs}. However, ACT efficacy could still be achieved with the transfer of a large number of transduced CTLs alongside vaccination or by reducing the time between tumour inoculation and ACT. These various models established platforms with which to assess the effect of genetically-modified CD8 T cells.

The creation of the pMKO.1 vector expressing a Thy1.1 reporter gene was achieved through a multistage cloning process. Although pMKO.1 GFP Thy1.1 was capable of transducing CD8 T cells, the efficiency levels achieved were low compared to the other empty vectors, with typically less than 20% co-expression of Thy1.1 and GFP. This co-expression was surprising, as a stop codon is present at the end of the Thy1.1 gene and therefore should have prevented the expression of the GFP. However, sufficient leaking through the stop codon or potentially internal ribosomal binding (although an IRES is not present) could potentially allow for co-expression of both Thy1.1 and GFP. The lower transduction efficiency achieved by the pMKO.1 GFP Thy1.1 vector was an impractical basis for further additions of shRNAs. However, future work may permit the removal of the GFP gene from the pMKO.1 GFP Thy1.1 vector through another multistage cloning process. This would consist of cutting the plasmid with the restriction enzymes ClaI and either EcoRV or NheI to remove the GFP gene, creating a new pMKO.1 Thy1.1 vector without the GFP. As a surface marker is a highly desirable trait to allow for the immunomagnetic purification, this project may be a worthwhile undertaking.

Although it wasn't feasible to use the pMKO.1 vector with Thy1.1, the pMKO.1 GFP vector showed adequate levels of transduction and was used for the incorporation of shRNA sequences. It is worth noting from a technical standpoint that the standard molar ratio of insert to vector (3:1) failed to yield positive ligations with many of the shRNA sequences. While the phosphorylation of annealed oligos would have increased the efficiency of this process, the risk of concatemer

formation would have also been raised greatly and was therefore avoided. To address this issue, an extremely high insert:vector ratio was used (1000:1) and achieved ligation for all constructs.

Taken together, the results here show the optimisation of protocols for OT-I T cell transduction and ACT against EG7 tumours with and without irradiation or vaccination strategies to achieve different levels of therapy. The retroviral assets constructed here allowed for the modification of CD8 T cells, the consequence of which is investigated in the following chapters.

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Chapter 4: Overexpressing transcription factors to promote memory CD8 T cell differentiation and augment ACT

4.1 Introduction

Pre-clinical models and retrospective analysis of clinical trials have demonstrated that the transfer of T cell populations containing higher proportions of T_{cm} phenotypes can enhance the efficacy of ACT therapy against cancer due their improved *in vivo* persistence and responsiveness compared to more terminally differentiated effector phenotypes [279, 283, 368, 369]. While the differentiation of CD8 T cells is a highly complex process, regulated by the strength and duration of signals emanating from numerous pathways, understanding the underlying molecular mechanisms of this process will provide rationales for enhancing ACT efficacy by manipulating factors that guide T cell fate and function.

Recent work has highlighted the importance of the PI3K/Akt signalling pathway in CD8 T cell differentiation. In a murine ACT model the treatment of TILs with Akti, a pharmacological inhibitor of Akt, allowed for the rapid *in vitro* expansion of cells in IL-2 while maintaining the functional and metabolic properties of memory CD8 T cells relative to cells cultured without Akti treatment [290, 370]. Following an ACT protocol with multiple rounds of *in vivo* restimulation, anti-tumour immunity was enhanced with the use of CD8 T cells that had been subjected to Akt inhibition during *ex vivo* expansion [290]. This illustrates the potential benefit of manipulating T cells by modulating signalling pathways, yet further work is required to characterise the precise molecular mechanisms underlying Akt-coordinated differentiation.

A consequence of inhibiting Akt in *ex vivo* expanded T cells is the sustained activity of FOXO1 and FOXO3 transcription factors. Although FOXO1 and FOXO3 possess many overlapping structural and functional features, they perform several distinct roles for T cells differentiation and survival (see **Table 4.1** and **Chapter 1.2.9**). For example, the inactivation of FOXO3 can promote the survival of CD8 T cells via reduced BIM mediated apoptosis [88] yet the disruption of FOXO1 activity hinders the ability of CD8 T cells to form functional memory and is linked to reduced Eomes expression [79, 81, 82]. Most of the research surrounding FOXO1 and FOXO3a in CD8 T cell immunity has utilised conditional knockouts within inflammatory infectious models. It is

therefore unknown how CD8 T cells will respond to FOXO manipulation in the context of cancer immunotherapy.

	FOXO1	**FOXO3a**
Survival	Helps survival by promoting CD127 [78].	Hinders survival BIM and PUMA [92].
Trafficking	Promotes CCR7 and KLF2 mediated upregulation of CD62L [76-79].	Promotes KLF2 mediated upregulation of CD62L [371].
Primary expansion	Does not affect expansion or differentiation [79, 82].	Sometimes limits expansion due to increased cell death [88].
Contraction following antigen clearance	Helps in the formation of functional memory [81].	Sometimes increases contraction due to cell death [92].

Table 4.1: Comparison between the known effects of FOXO1 and FOXO3a. These observations are generated from these studies utilising conditional T cell FOXO knockouts within LM or LCMV infection models.

4.1.1 Constitutive activation of FOXO proteins

As well as the disruption of proteins the function FOXOs can be investigated through the introduction of CA FOXO1 or CA FOXO3a, which are resistant to Akt-mediated phosphorylation. The Akt insensitivity is achieved by introducing three alanine substitutions, one at each Akt phosphorylation sites previously mentioned, producing a FOXO AAA mutant [372, 373]. Preventing the phosphorylation of FOXO proteins prohibits their 14-3-3 protein-mediated egress from the nucleus and therefore maintain constant transcriptional activity alongside activated Akt.

Previous investigations have demonstrated that double negative thymocytes transduced with CA FOXO3 have lower levels of accumulation within the pre-T cell compartment, linked to increased BIM-mediated apoptosis [374]. However, *in vitro* experiments have shown that CA FOXO3 in CD8 T cells results in upregulation of CD62L, which is maintained even during expansion in IL-2 [371]. Therefore, the CA FOXO3a appears to play a role in both limiting survival yet also promoting aspects of a memory T cell phenotype. With these are conflicting effects, it is of interest to elucidate whether the function memory T cells is enhanced by the presence of CA FOXO3a and if this will compensate for potently increased levels of apoptosis in these cells.

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While it is clear that the physiological activity of FOXO1 is necessary for the formation of functional memory T cells, CA FOXO1 is yet to be investigated in the context of CD8 T cell differentiation and if memory is promoted, what impact this may have on the efficacy of ACT. Recently, a knock-in FOXO1 AAA ($Foxp3^{cre}$) mouse model has been used to explore the role of FOXO1 in T_{reg} [375]. The results demonstrated that CA FOXO1 favours a resting T_{reg} state with an increased turnover rate and enhanced expression of CD62L. Consistent with higher CD62L expression, it was found that CA FOXO1 favoured T_{reg} cell trafficking to lymphoid organs at the cost of peripheral surveillance, ultimately resulting in CD8 T cell-mediated autoimmunity. It is therefore of interest to explore whether CA FOXO1 regulates the localisation of CD8 T cells in a similar way and what effect this will have on differentiation and function.

4.1.2 Project Aims

Here, I used a retroviral a transduction strategy to introduce a CA FOXO1 or CA FOXO3a into *in vitro* activated OT-I CD8 T cells to delineate their effect on proliferation, differentiation, and effector function within *in vitro* and *in vivo* settings. The impact of CA FOXO1 on CD8 T cell function was then assessed in a preclinical ACT model. Finally, the consequence of overexpressing Eomes, another transcription factor known to promote T_{cm} formation (See **Chapter 1.2.8.1** for a full introduction), in CD8 T cells was also evaluated.

4.2 Results

Following the validation of the retroviral vectors described in **Chapter 3.2.4**, the phenotypic and functional impact of CA FOXO1 or CA FOXO3a was evaluated *in vitro*. Transduced OT-Is were expanded in IL-2 for three days, generating CD8 T cells with an effector-like profile [376]. IL-2 signalling through the PI3K/Akt pathway ensured consistent downregulation of intrinsic FOXO1 and FOXO3a activity for the CD8 T cells transduced with the empty vector [377]. The results here demonstrated that CD8 T cells transduced with CA FOXO1 or CA FOXO3a vectors substantially enhanced levels of FOXO1 and FOXO3a respectively, thereby validating these retroviral vectors (**Figure 4.1A**). Phenotypically, the expression of CA FOXO1 and CA FOXO3a caused upregulation of the memory marker CD62L (**Figure 4.1B**). This effect appeared to be far greater for the T cells expressing CA FOXO1, although it is possible that different levels of retroviral integration or protein expression between the two CA vectors may have influenced this result. The CA FOXO vectors expression also made no impact on the accumulation of CD8 T cells during the IL-2 mediated expansion (**Figure 4.1C**). Together, these data validate the overexpression of FOXO1 or FOXO3a in CD8 T cells can be used to promote a feature of the memory phenotype without limiting IL-2 mediated expansion *in vitro*.

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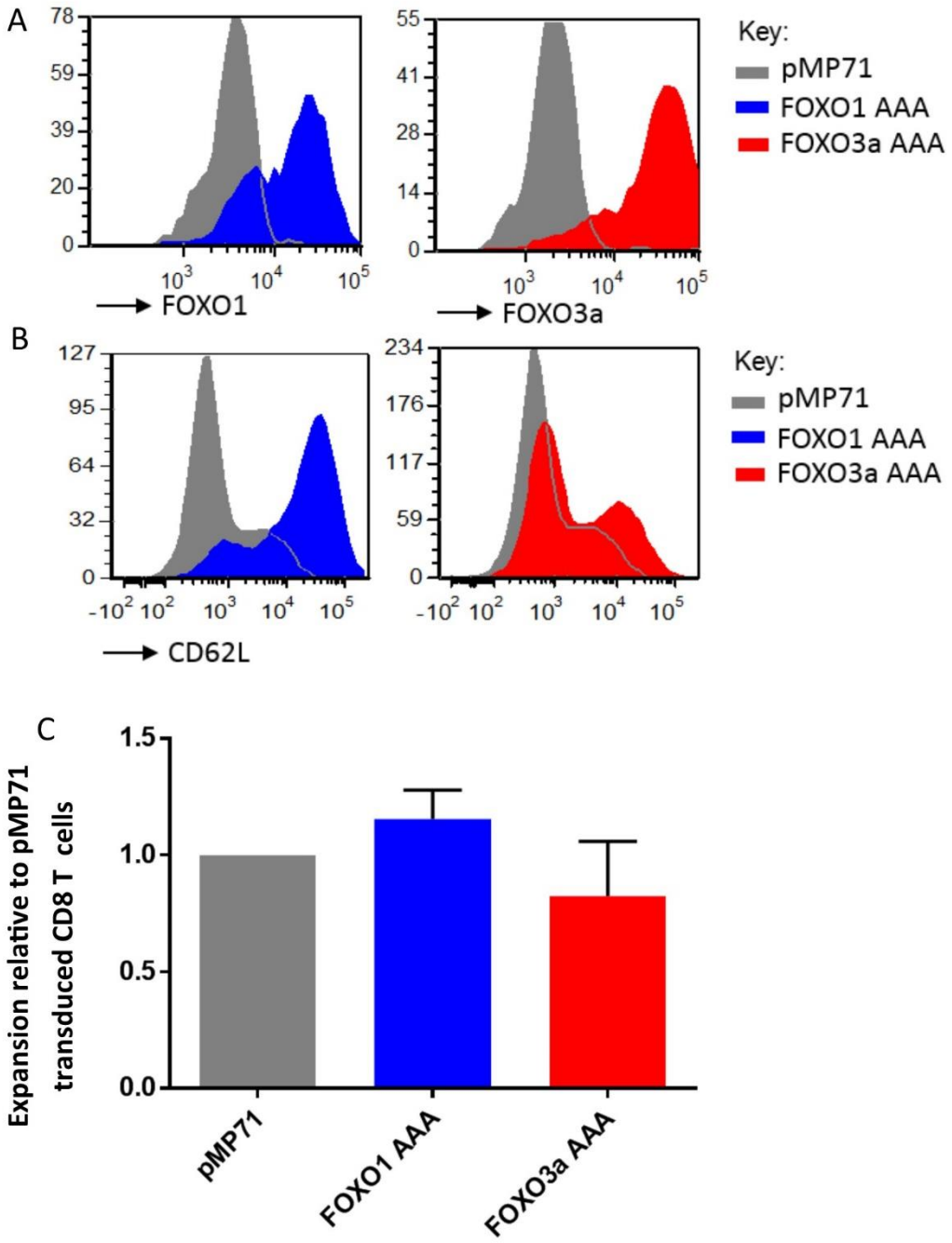

Figure 4.1: Evaluating CA FOXO1 and CA FOXO3a in CD8 T cells. Naive OT-I splenocytes were stimulated with Con-A (5μg/ml) and IL-7 (1ng/ml) for 24 hours prior to transduction with pMP71, FOXO1 AAA, or FOXO3a AAA retrovirus. The transduced cells were then expanded in IL-2 (10ng/ml) for three days. A, Intracellular expression of FOXO1 or FOXO3a was assessed by flow cytometry, gating on transduced GFP^+ CD8 T cells. B, Surface CD62L expression as assessed by flow cytometry. C, The expansion of transduced cells was calculated relative to the pMP71 control on day three of the IL-2 expansion *in vitro*. Data are representative or pooled from at least two independent experiments.

As CA FOXO1 and CA FOXO3a enhanced CD62L expression, the next experiment aimed to elucidate whether aspect survival, phenotype, recall ability, and localisation of transduced CD8 T cells would be affected *in vivo*. With upregulated CD62L, it was possible that improved homing to lymphoid organs could facilitate increased exposure to homeostatic cytokines to promote the survival transferred CD8 T cells. Following the standard transduction procedure, OT-I T cells expressing CA FOXO1 or CA FOXO3a were transferred to WT C57BL/6 mice where their frequency and phenotype was monitored from peripheral blood samples. Relative to co-transferred non-transduced cells or the transfer of control transduced cells, the presence of CA FOXO1 did not improve CD8 T cell engraftment or secondary expansion following vaccine rechallenge with SIIN and αCD40 administered 24 days post transfer (**Figure 4.2A and B**). Additionally, CA FOXO3a did not affect the engraftment of CD8 T cells but did cause a significant reduction in the frequency of cells seen following the vaccine rechallenge (**Figure 4.2A and B**). To evaluate whether the expression CA FOXOs affected the localisation of CD8 T cells, lymphoid and non-lymphoid tissues were harvested 21 days after the secondary rechallenge. Here it was shown that the presence of CA FOXO1 accounted for a significant (two-fold) reduction in the localisation of CD8 T cells in the lungs (**Figure 4.2A**). However, despite the fact that CA FOXO1 maintained significantly higher expression of T_{cm} markers, such as CD62L and CD127, the accumulation of cells expressing this factor was not enhanced in the lymph nodes (**Figure 4.3B**). Additionally, the presence of either CA FOXO1 or CA FOXO3a significantly reduced the proportion of KLRG1^{hi} CD8 T cells present in the lungs and spleen (**Figure 4.3B**). A complementary strategy was also used to assess the phenotype of the secondary memory cells based on CXCR3 and CD43 markers [42, 378, 379]. The CA FOXO1 significantly reduced the proportion of T_{em} associated subset, CXCR3^{lo}, which was compensated by a reciprocal increase in the T_{cm} associated subset CD43^{lo} CXCR3^{hi} for each tissue analysed (**Figure 4.4**). These data demonstrated that CA FOXO1 drove the phenotypic characteristic of $T_{cm,}$ but this was not associated with enhanced engraftment, response to rechallenge, or localisation to lymphoid organs. The CA FOXO3a significantly reduced the frequency of KLRG1^{hi} CD8 T cells in the lungs (**Figure 4.3B**) and gave a slight enhancement to the CD43^{lo} CXCR3^{hi} subsets in the spleen and lungs (**Figure 4.4**) yet did not impact other phenotypic trails. Compared to CA FOXO3a, the introduction of CA FOXO1 produced a greater proportion of cells with a T_{cm} profile without compromising the frequency of cells during the secondary response. As it provided more positive traits for augmenting ACT, CA FOXO1 became the sole focus of the following experiments.

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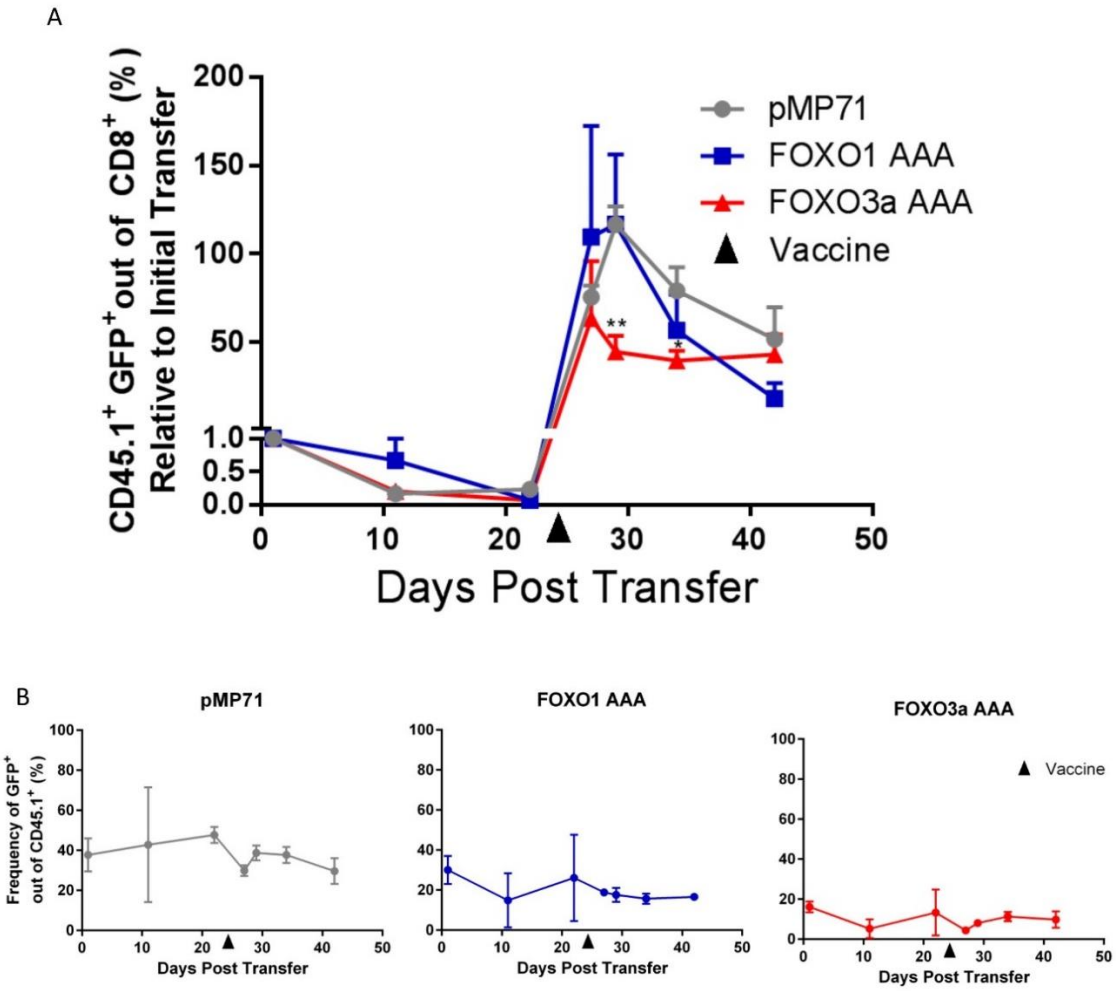

Figure 4.2: The effect of CA FOXO1 or CA FOXO3a on the in vivo engraftment and secondary response of CD8 T cells. Naive OT-I splenocytes were activated and transduced with pMP71, FOXO1 AAA, or FOXO3a AAA retrovirus, and then cultured for 48 hours in IL-7 (20ng/ml) as opposed to the standard IL-2 culture. A and B, $1.25x10^6$ CD8 T cells were transferred into WT C57BL/6 mice. The persistence of cells was monitored from peripheral blood samples, assessing $CD45.1^+$ GFP^+ by flow cytometry. On day 24 post transfer, the mice were challenged by a vaccine (SIIN (30nM) and αCD40 (50μg)), administered by i.v. tail injection as indicated by the arrow. Data are from a single experiment with three mice per group.

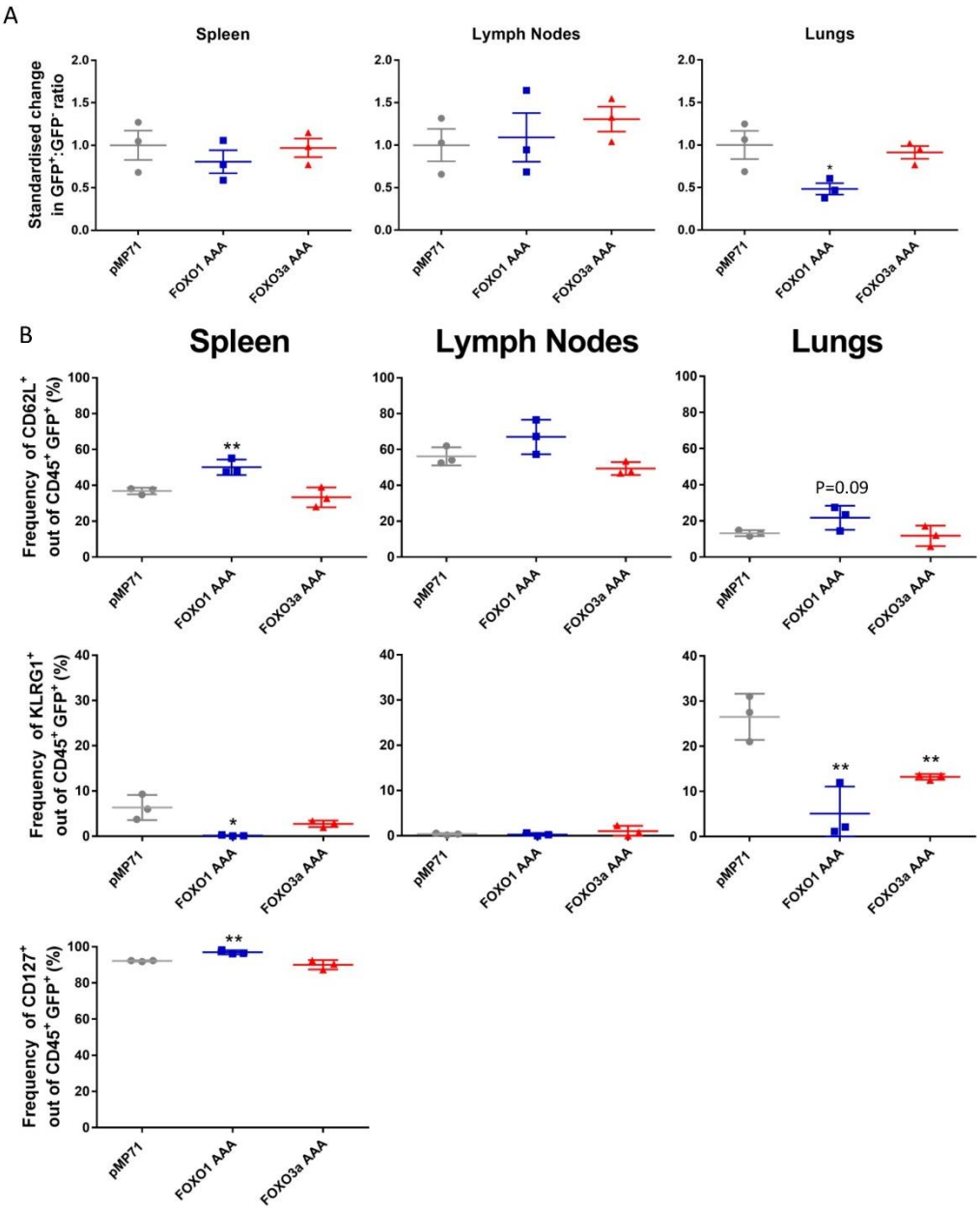

Figure 4.3: The effect of CA FOXO1 and CA FOXO3a on the localisation and classic differentiation phenotype of CD8 T cells. OT-I cells were transduced with pMP71, FOXO1 AAA, or FOXO3a AAA retrovirus, and transferred to mice described in **Figure 4.3.** 24 days post transfer, the mice were challenged by a vaccine (SIIN (30nM) and αCD40 (50µg). The spleen, inguinal lymph nodes and lungs of these mice were then harvested 21 days post vaccination. A, Flow cytometry was used to assess the change in GFP^+:GFP^- ratio out of $CD45.1^+$ compared to the initial GFP^+:GFP^- ratio prior to adoptive transfer and was then standardised against the average change from the control pMP71 group. B, The phenotype of transferred cells in the organs was assessed by flow cytometry. These data are from a single experiment with three mice per group.

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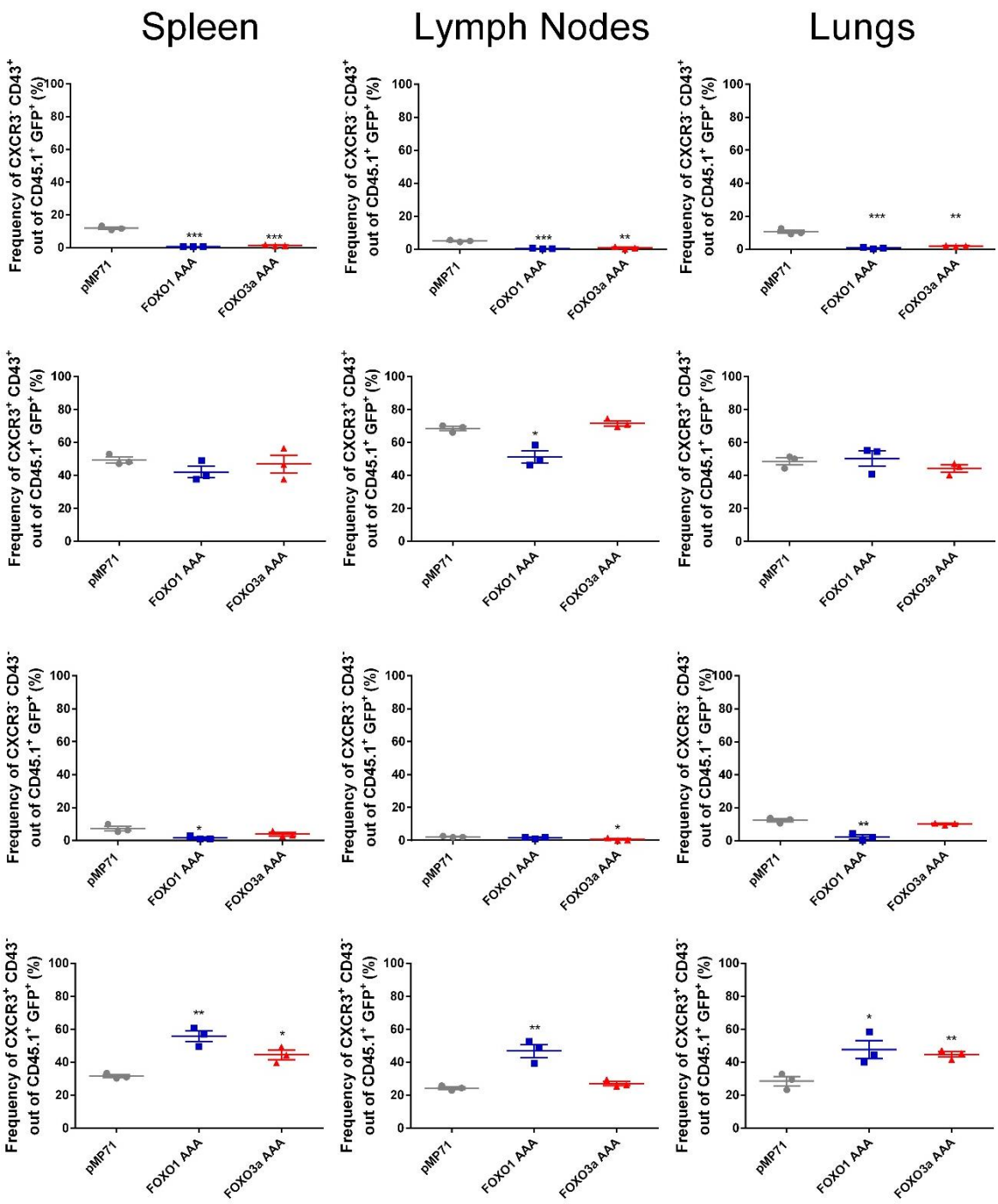

Figure 4.4: The effect of CA FOXO1 or CA FOXO3a on the CXCR3/CD43 phenotypic profile of CD8 T cells. OT-I cells were transduced with pMP71, FOXO1 AAA, or FOXO3a AAA retrovirus, and transferred to mice described in **Figure 4.3.** 24 days post transfer, the mice were challenged by a vaccine (SIIN (30nM) and αCD40 (50µg). The spleen, inguinal lymph nodes and lungs of these mice were then harvested 21 days post vaccination. The phenotype of transferred cells in the organs was assessed by flow cytometry. These data are from a single experiment with three mice per group.

The sub-lethal irradiation of mice creates a lymphopenic environment, in which the levels of lymphopenia-induced proliferation (LIP) are positively correlated with the expression of CD127 on adoptively transferred CD8 T cells (50). As my previous results demonstrated that CA FOXO1 did not impact the engraftment of cells into immune competent hosts, I postulated that the sub-lethal irradiation could create an environment conducive to improved engraftment of CD8 T cells with CA FOXO1. Here, transduced OT-I splenocytes were purified and expanded in IL-2 for five days, at which point CD127 expression was not detectable for the control cells, but was expressed by 21% of the cells possessing CA FOXO1 (**Figure 4.5A**). Following this expansion, the purified cells were transferred in WT C57BL/6 mice that had been sub-lethally irradiated (5.5Gy) (**Figure 4.5B**). The presence of CA FOXO1 significantly enhanced the engraftment of CD8 T cells into lymphopenic mice, demonstrating consistently high frequencies until day 23 post transfer (**Figure 4.5C**).

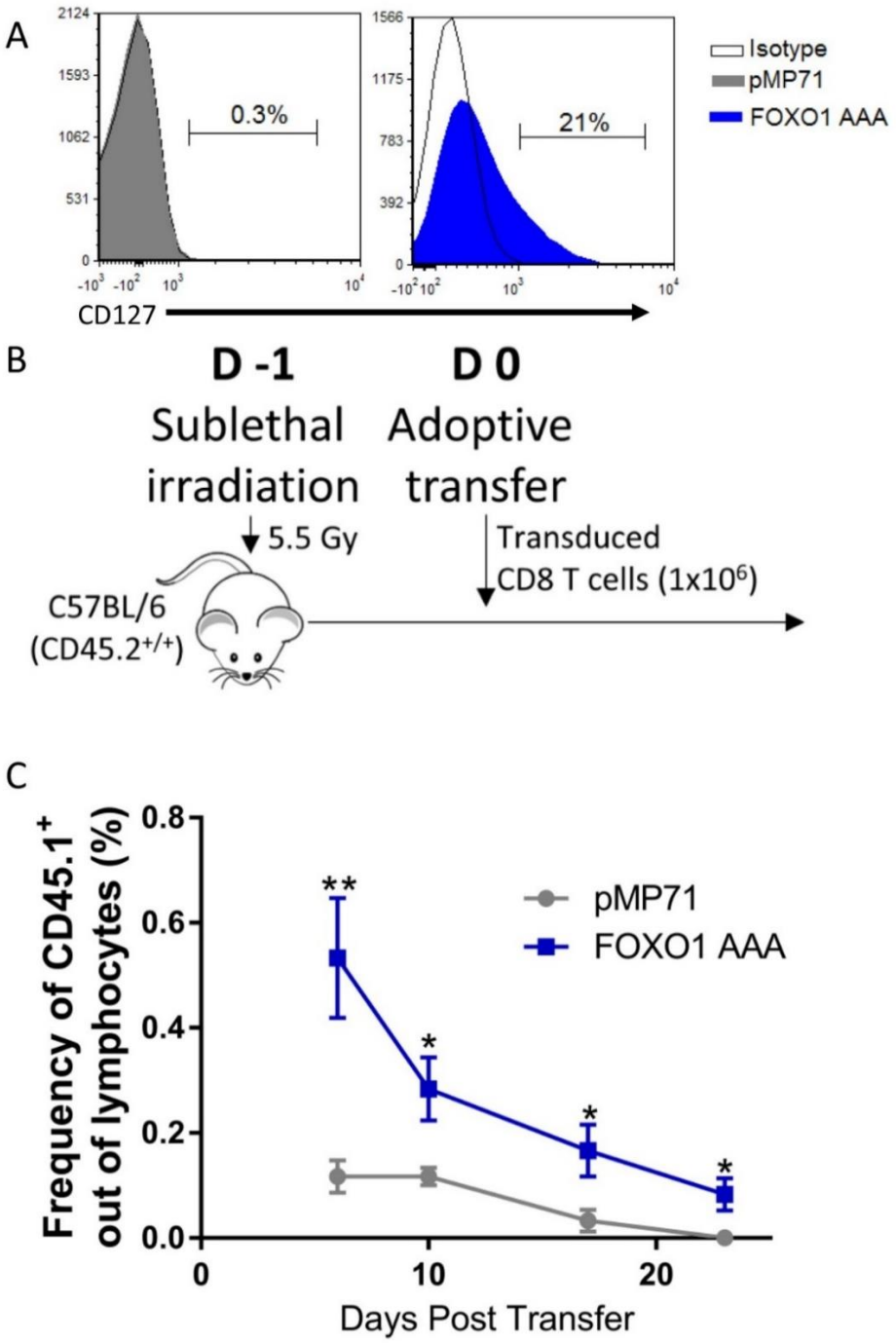

Figure 4.5: The impact of CA FOXO1 on the engraftment of CD8 T cell into lymphopenic hosts. OT-I splenocytes transduced with pMP71 or FOXO1 AAA and sorted by FACS (GFP^+ $CD4^-$) before being expanded for 5 days in IL-2 (10ng/ml). A, Following the expansion, the expression of CD127 on transduced CD8 T cells was assessed by flow cytometry. B, Schematic detailing the transfer protocol. WT C57BL/6 mice were subject to irradiation (5.5Gy), which was followed one day later by the transfer of the *in vitro* expanded transduced CD8 T cells ($1x10^6$). C, Frequency of transferred cells was assessed in peripheral blood samples, analysed by flow cytometry. These data are from one experiment with five mice per group.

As CD8 T cells expressing CA FOXO1 showed enhanced phenotypic characteristics of memory cells and improved engraftment in a lymphopenic environment, the functional ability of these cells to mediate anti-tumour immunity was assessed in a model of ACT featuring the sublethal irradiation preconditioning described in **Chapter 3.2.1**. CD8 T cells expressing CA FOXO1 were purified and expanded in IL-2 for five days prior to adoptive transfer to sublethally irradiated mice bearing tumours. In line with the previous data, cells expressing CA FOXO1 showed a similar *in vitro* accumulation compared to the control transduced cells, 19.5-fold versus 18.5-fold expansions respectively, during the five-day culture (**Supplementary figure 4.1A**). Following this expansion, the phenotype of cells was assessed, demonstrating consistent results with previous with enhanced CD62L and CD127 expression by the cells possessing CA FOXO1 (**Supplementary figure 4.1B**). This analysis also showed that CA FOXO1 expression caused a slight downregulation of T-bet, a transcription factor associated with effector differentiation, and the cytotoxic molecule granzyme B (**Figure 4.6A**). Furthermore, to test the functional capabilities, the transduced OT-I cells were stimulated with a high-affinity peptide (SIIN) and cytokine production was measured. The results here showed that CA FOXO1 mediated a slight reduction in the ability of cells to express IFNγ and IL-2 (**Figure 4.6B**). Sub-lethally irradiated WT mice were inoculated with EG7 tumours, which were left to establish for five days before ACT treatment (**Figure 4.6C**). While the transfer of transduced CD8 T cells produced a significant delay in tumour growth, the presence of CA FOXO1 was shown to limit this effect (**Figure 4.6D**). Additionally, the CA FOXO1 transduced cells did not mediate the improved survival as seen in the control group (**Figure 4.6D**). These data suggest CA FOXO1 negatively influences the ability of CD8 T cells to mount an effective anti-tumour response.

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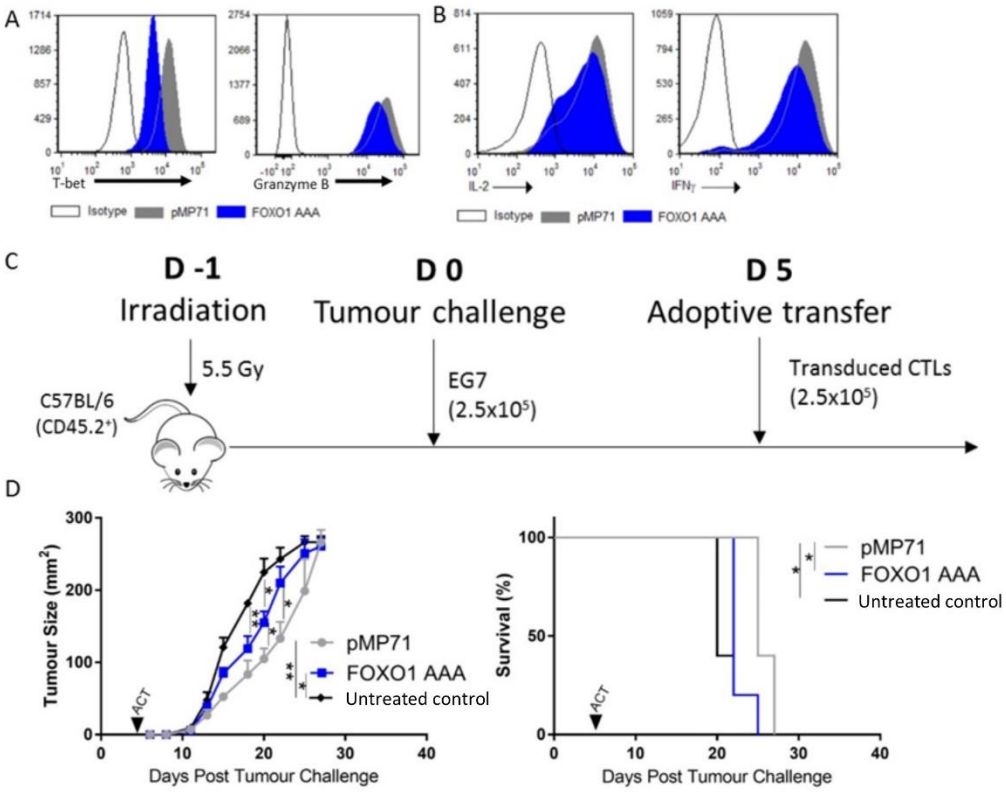

Figure 4.6: The effect of CA FOXO1 on ACT therapy against tumours. Naïve OT-I splenocytes were activated and transduced with FOXO1 AAA or pMP71 retrovirus following the standard protocol. The transduced CD8 T cells were purified by FACS (GFP^+ $CD4^-$) and expanded for five days in IL-2 (10ng/ml) refreshing cytokine daily. A, Following the expansion, the phenotype of the transduced cells was assessed by flow cytometry. B, The *in vitro* expanded cells were cultured for four hours with splenocytes, Golgi-plug and peptide (10nM SIIN). The cytokine production was then assessed by flow cytometry. C, Schematic detaining ACT protocol. 24 hours after sublethal irradiation, WT C57BL/6 mice were challenged with EG7 tumour cells ($2.5x10^5$) via s.c. injection. Five-days post tumour challenge the mice were treated with the *in vitro* expanded transduced OT-I cells ($2.5x10^5$). D, Tumour sizes were monitored (left panel) until the terminal endpoint of the experiment (right panel). Data from A and B are representative of at least two independent experiment. Data from D are from one experiment with 5 mice per group. For the left panel of D, the statistics shown between data points are two-way student T-test, while the statistics between the legends are calculated as two-way ANOVAs.

Next, an experiment was conducted to assess if CA FOXO1 in CD8 T cells would be more responsive to vaccinations within the ACT model featuring sublethal irradiation. Purified transduced OT-I CD8 T cells expressing CA FOXO1 were cultured for 5 days in IL-2 before being transferred into irradiated mice bearing an established EG7 tumour. The mice were then vaccinated with SIIN peptide on days-1 and 11 post adoptive transfer (**Figure 4.7A**). The results demonstrate that the presence of CA FOXO1 in CD8 T cells hindered ACT efficacy compared to the

transfer of control transduced cells, reflected by significantly decreased ability to control tumour growth and a shorter survival duration for the mice (**Figure 4.7B-E**). Indeed, at day-11 post tumour, the transfer of control transduced cells showed significantly smaller tumour burdens compared to the mice receiving CA FOXO1 transduced cells or the naïve control mice without any therapy (**Figure 4.7C**). Monitoring the transferred cells from peripheral blood samples revealed that the presence CA FOXO1 significantly increased the responsiveness of cells to the peptide vaccination as shown by the increased number of cells at day 14 post transfer (**Figure 4.7F and G**). As shown previously, the expression of CD62L as well as the frequency of cell with a memory profile, $CD127^{+}$ $KLRG1^{-}$, were significantly increased by the presence of CA FOXO1 (**Figure 4.8A and B**). Additionally, in this setting T-bet expression was downregulated by CA FOXO1 (13 days post transfer), while not influencing Eomes expression (**Figure 4.8A and B**). Finally, at day 8 post transfer, CA FOXO1 was shown to significantly decrease the expression of the cytotoxic molecule, granzyme B (**Figure 4.8A and B**). Taken together, these data illustrate that the CA FOXO1 can promote the responsiveness to a weak peptide vaccination in this lymphopenic setting, yet also reduced the cytotoxic potential of CD8 T cells, which may have contributed to the resulting decrease in anti-tumour immunity.

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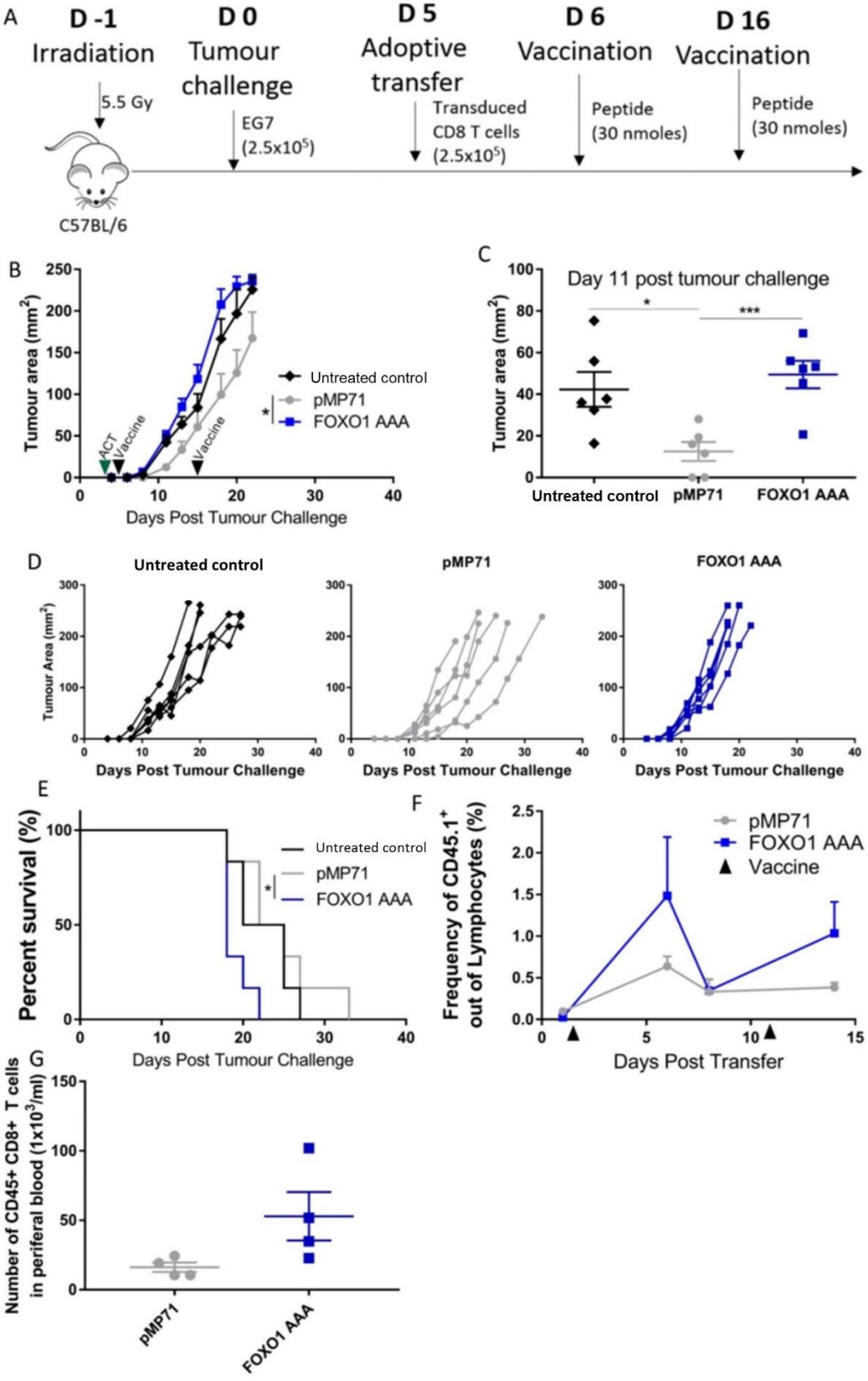

Figure 4.7: The effect of CA FOXO1 on CD8 T cell-mediated tumour control during ACT with a vaccination strategy. Naïve OT-I splenocytes were activated, transduced with pMP71 or FOXO1 AAA retrovirus, sorted by FACS (GFP^+ $CD4^-$), and cultured in IL-2 for five days refreshing cytokine daily. A, Schematic detailing the ACT protocol. WT C57BL/6 mice were irradiated (5.5Gy) then

challenged with EG7 cells ($2.5x10^5$) by s.c. injection on the following day. Five days post tumour challenge the mice were treated with the transduced CTLs. On day-six and sixteen post tumour challenge the mice were vaccinated with peptide (SIIN 30nM). B-D the tumour size was monitored over time. E, The survival of the mice until terminal end-point of the experiment. F, The frequency of transferred cells was evaluated in peripheral blood samples using flow cytometry. G, Total counts from blood were used alongside the frequency of $CD45.1^+$ out of viable cells, measured by flow cytometry, to calculate numbers for transferred cells present at day 14 post adoptive transfer. This experiment was performed once with six mice per group.

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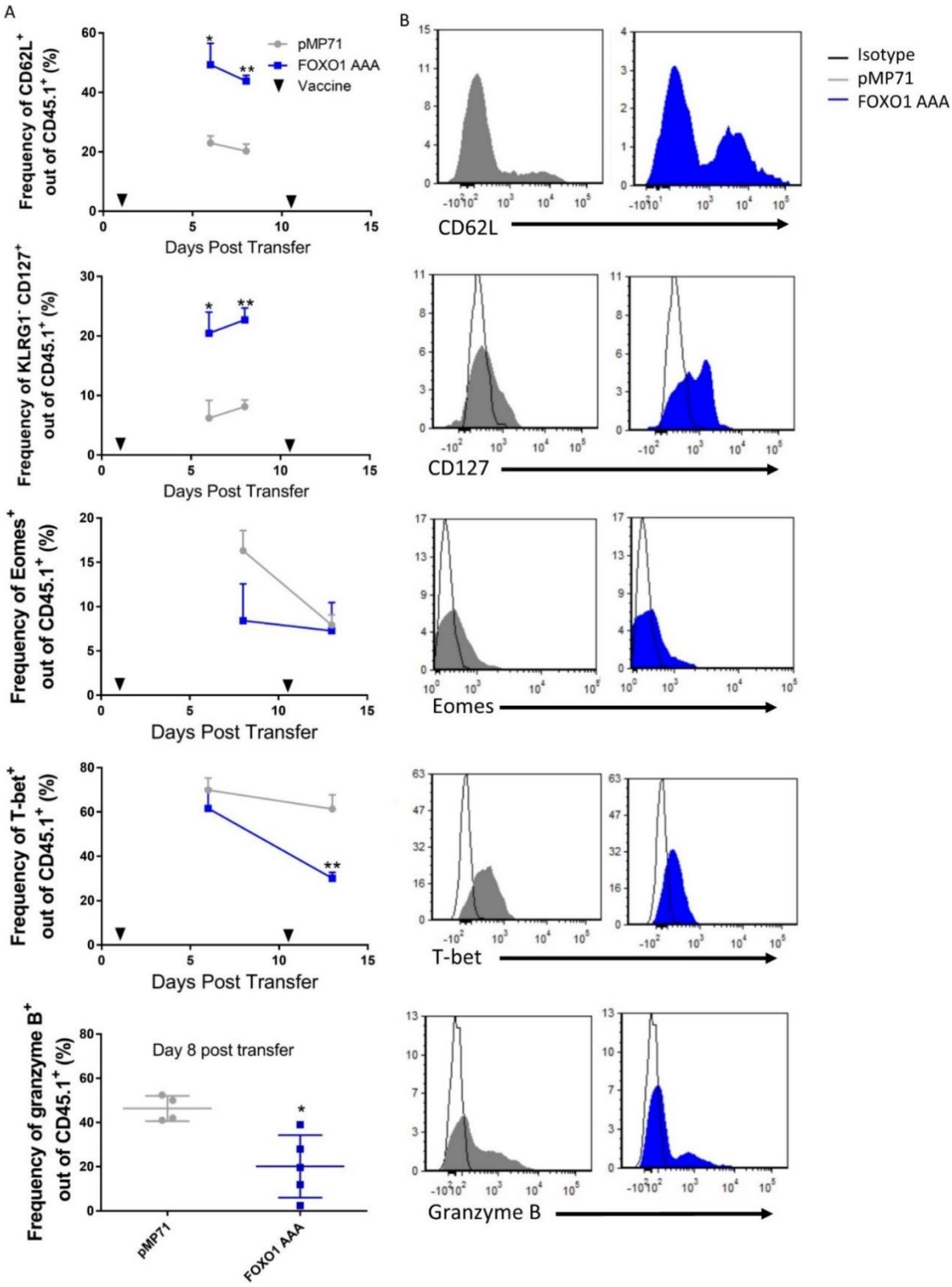

Figure 4.8: The effect of CA FOXO1 on the in vivo profile of transferred CD8 T cells during ACT alongside vaccination. Data continue from the experiment described in **Figure 4.8.** Briefly, OT-I cells were transduced with FOXO1 AAA or pMP71 retrovirus, purified and transferred to irradiated mice and bearing tumour, which the received subsequent vaccination on day 1 and 11 post transfer peptide (SIIN 30nM). A and B, flow cytometry was used to assess phenotype of the transferred cells from peripheral blood samples. B, Representative histograms are shown

from day 8 post transfer for all parameters, except T-bet and Eomes, which are shown for day 13 post transfer.

As the constitutive activation of FOXO1 promoted the phenotype of central memory but appeared to limit the ability of CD8 T cell to respond to tumours *in vitro*, it was next of interest to explore whether the transcription factor Eomes could be used to manipulate memory formation without impacting cytotoxic capabilities. Murine models have shown that the knockout of Eomes negatively impacts memory formation as well as the expression molecules such as IFNγ [58]. Therefore, experiments were conducted to elucidate whether improving memory formation and effector function could be achieved by overexpressing Eomes in CD8 T cells. Firstly, an Eomes overexpression vector was generated, as detailed in **Chapter 3.4**. To validate the function of the pMP71-Eomes vector, transduced OT-I T cells were purified by FACS and expanded in IL-2 for five days wherein the levels of Eomes were shown to be clearly upregulated (**Figure 4.9A**). The impact of overexpressing Eomes during IL-2 or IL-15 mediated differentiation *in vitro* was also assessed. When CD8 T cells were expanded in IL-2 to generate a SLEC profile, the Eomes overexpression resulted in the upregulation of the memory marker CD62L and the downregulation of the activation and effector markers T-bet, CD25, CD69, and granzyme B (**Figure 4.9B**). When CD8 T cells were expanded in IL-15 to generate an MPEC profile, Eomes overexpression again resulted in the upregulation of CD62L but was also shown to mediate a slight increase in granzyme B and CD44 expression, which are indicative of effector differentiation (**Figure 4.9B**). The FACS purified OT-I CD8 T cells, transduced with pMP71-Eomes were expanded for 5 days in IL-2 before being cultured with CD8 depleted irradiated splenocytes for four hours in the presence of peptides (SIIN, SIIQ, and SIIT) covering a range of affinities and concentrations to assess the ability of these cells to produce cytokines. The results here show that Eomes overexpression resulted in a global downregulation in IFNγ, TNFα, and IL-2 expression (**Figure 4.9C**). With high levels of SIIN stimulation, the cells overexpressing Eomes achieved maximal expression of TNFα. Yet for all other conditions, the overexpression of Eomes resulted in lower levels of cytokine production. In particular, the loss in IL-2 expression was very striking, showing a four-fold reduction in expression levels at the higher levels of peptide stimulation.

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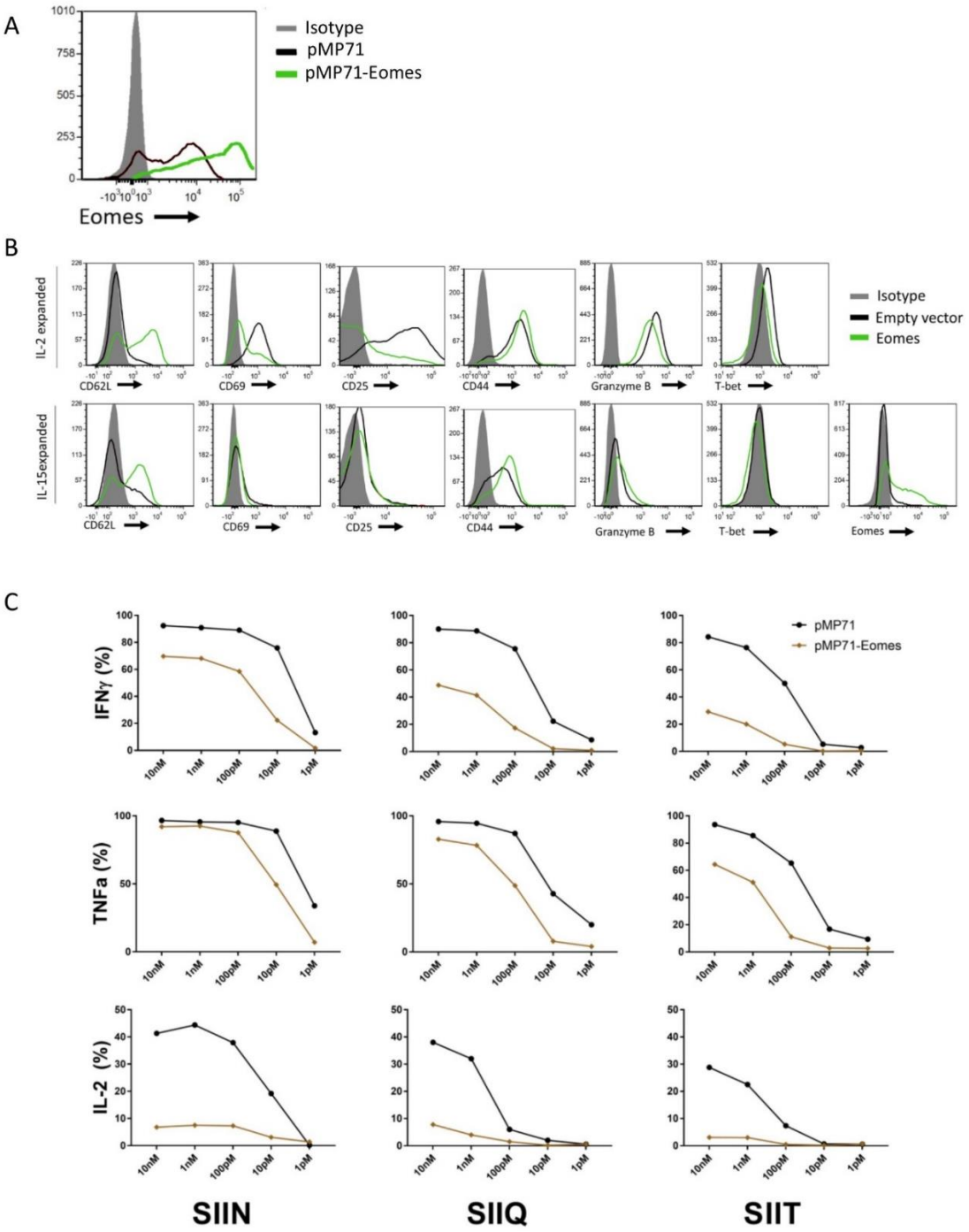

Figure 4.9: Validation and characterisation of the Eomes overexpression vector in CD8 T cells. Naïve OT-I splenocytes were activated and transduced with retrovirus, pMP71 or pMP71-Eomes, following the standard protocol. The transduced cells were purified by FACS (GFP^+) and expanded for five days in IL-2 (10ng/ml) (A, B (top panel), and C) or IL-15 (15ng/ml) (B (bottom panel)) refreshing cytokine daily. A and B, Following the *in vitro* expansion, the phenotype of transduced cells was assessed by flow cytometry. Histograms are representative of two independent experiments. C, Following the expansion in IL-2, the transduced cells were cultured for four hours with CD8 depleted irradiated splenocytes, Golgi-plug and the peptide

indicated. The cytokine production was then assessed by flow cytometry. Data are from one experiment.

To characterise the impact of Eomes overexpression in an *in vivo* setting, FACS purified OT-I CD8 T cells, transduced with pMP71-Eomes or pMP71, were expanded for 5 days in IL-2 then transferred into WT C57BL/6 mice (**Figure 4.10A**). One-day post transfer the frequency of CD45.1$^+$ cells in the peripheral blood was significantly reduced by the overexpression of Eomes (**Figure 4.10B**). However, 24 days post transfer, a vaccination (SIIN and αCD40) resulted in equivalent frequencies at the peak secondary response for cells overexpressing Eomes compared to the control group (**Figure 4.10B**). At the peak of the secondary response organs were taken from 3 out of the 6 mice for further analysis of differentiation and localisation (**Supplementary figure 4.2)**. In the remaining mice, contraction of cells following secondary expansion was shown to be unaffected by Eomes overexpression (**Figure 4.10B**). Except for day 23, one day before vaccination, the levels of CD62L expression were observed as being significantly increased the transferred T cells overexpression Eomes (**Figure 4.10C**). Additionally, the overexpression of Eomes resulted in significant increased frequencies of CD8 T cells expressing KLRG1 in the peripheral blood following vaccination, with more than a four-fold increase at the peak of the secondary response, while no effect was seen on CD127 expression (**Figure 4.10D and E**). At the peak of the response, splenocytes were restimulated with SIIN peptide, to measure the ability of these transferred cells to produce cytokines. The expression of IFNγ and TNFα were not affected by the overexpression of Eomes, yet there was a significant reduction in the frequency of cells expressing IL-2 (**Figure 4.11A**). The pMP71-Eomes transduced cell were shown to retain dramatically higher levels of Eomes, which was present in 65% of cells versus 20% for the control group (**Figure 4.11B**). This coincided with a significant loss of T-bet expression, but the levels of granzyme B were unaffected (**Figure 4.11B**). Interestingly, Eomes overexpression did not significantly impact the total number or frequency of transferred cells localised in the spleen, lymph nodes, or lung but impact these cells phenotype (**Figure 4.11C** and **Figure 4.12A**). For example, the overexpression of Eomes resulted in a significantly higher frequency of CD127$^+$ KLRG1$^-$ CD8 T cells in the lungs, but significantly less of this same subset in the lymph nodes (**Figure 4.12A**). A universal trait across all organs was the finding that Eomes overexpression caused a significant increase in the proportion of cells expressing CD62L and KLRG1, the latter showing at least a seven-fold increased within each organ (**Figure 4.12A**). Interestingly, the overexpression of Eomes resulted in a novel phenotype of cells, CD62L$^+$ KLRG1$^+$, comprising 27% of the transferred cells in the lymph nodes (**Figure 4.12A**). Indeed, this phenotype was not observed in the small number of non-transduced

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cell in the pMP71-Eomes group or within the control transduced cell population (**Figure 4.12A and B**). Taken together, the overexpression of Eomes in CD8 T cells favoured phenotypic traits for effector and memory cells at various stages of the *in vitro* expansion and *in vivo* restimulation. However, no difference in the expansion or localisation seen from cells overexpressing Eomes and a consistent reduction in the ability of CD8 T cells to produce IL-2 upon stimulation was observed. As such, the overexpression of Eomes was not investigated further.

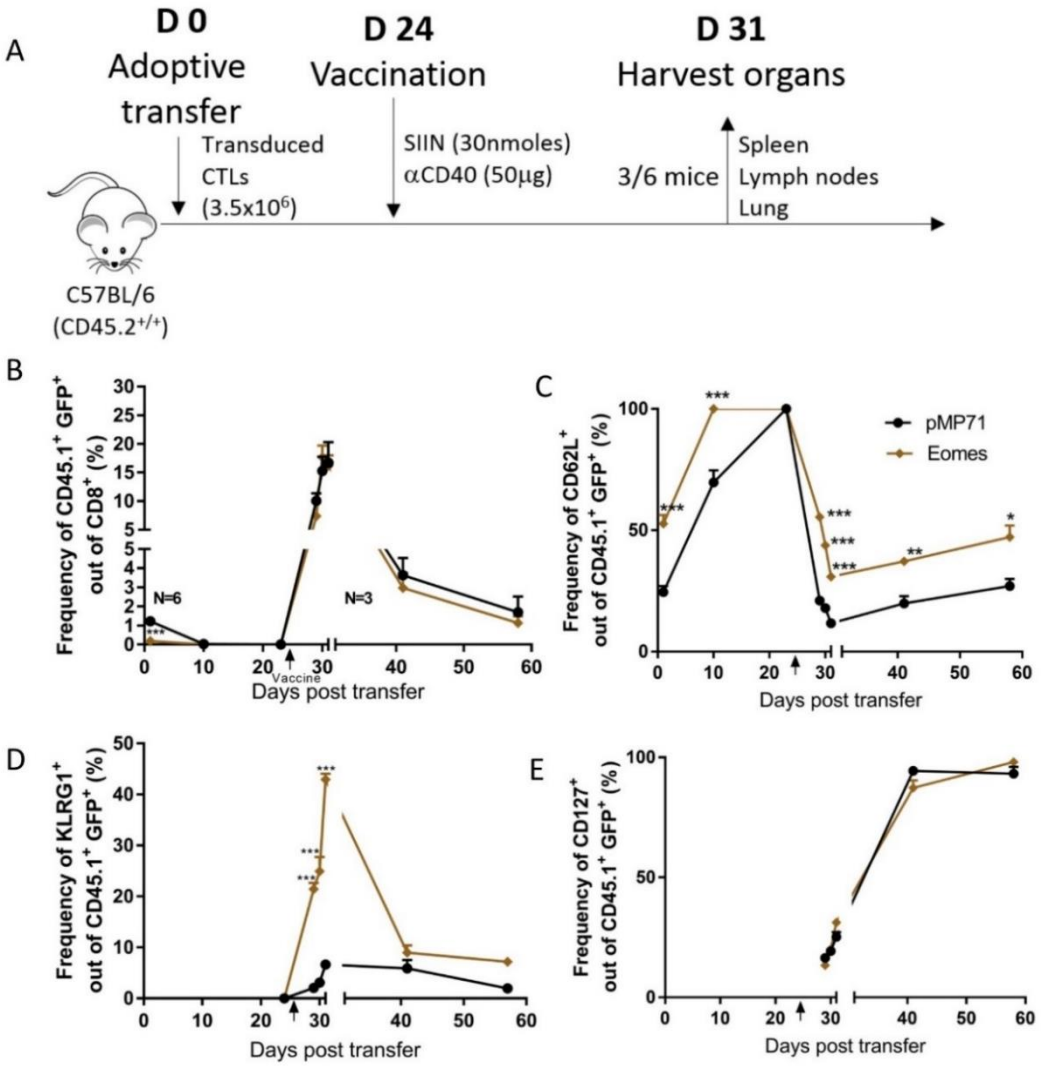

Figure 4.10: The effect of Eomes overexpression on the response of CD8 T cells to an in vivo rechallenge. Naïve OT-I splenocytes were activated and transduced with retrovirus, pMP71-Eomes or pMP71 vectors, following the standard protocol. The transduced CD8 T cells were purified by FACS (GFP^+ $CD4^-$) and expanded for five days in IL-2 (10ng/ml), refreshing cytokine daylily. A, A schematic details adoptive transfer and vaccination strategy. Briefly, following *in vitro* expansion, $3.5x10^6$ transduced cells were transferred to WT C57BL/6 mice, which were immunised 24 days later with SIIN (30nM) and αCD40 (50μg). Seven days after the rechallenge the spleen, liver, and lymph nodes of three out of the six mice were taken for further analysis. B-E, the frequency and phenotype of the transferred cells was assessed from peripheral blood samples by flow cytometry. This experiment was performed once with six mice in the experiment until day 31 and then three remaining mice for day 31 onwards.

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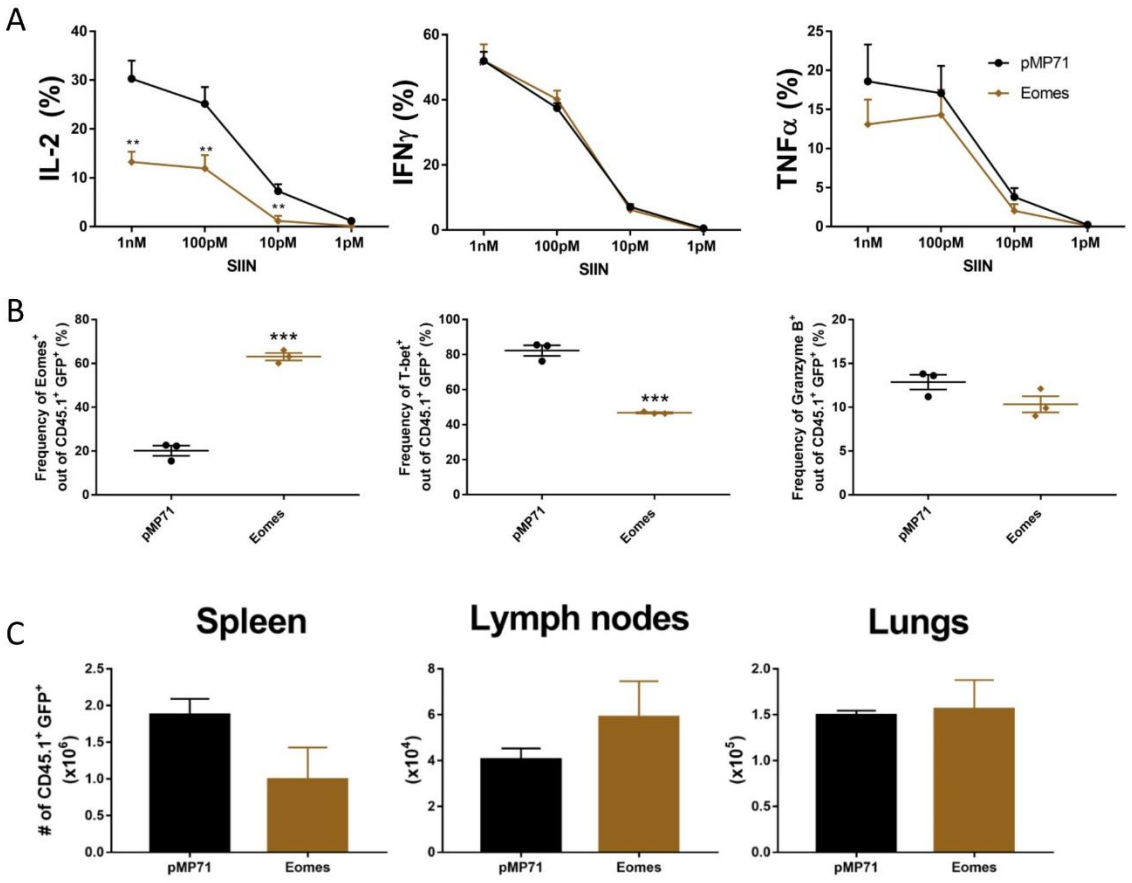

Figure 4.11: Function, differentiation, and localisation of CD8 T cells overexpressing Eomes during a secondary response. Continuing from the experiment established in **Figure 4.11**. On day seven post vaccine rechallenge, the spleen, lymph nodes, and lung of mice were harvested from mice (**Supplemental figure 4.2**). A, Splenocytes were restimulated with the peptide (SIIN) at the concentration indicated alongside Golgi Plug for four hours, at which point cytokine production of the transduced cells assessed by flow cytometry gating on the CD45.1^{+} population. B, The phenotype of the transferred cells in the spleen was assessed by flow cytometry. C, The total number of transferred cells in the spleen, lymph nodes and lungs was calculated from total count and the frequency of CD45.1^{+} out of viable cells measured by flow cytometry. This experiment was performed once with three mice per group.

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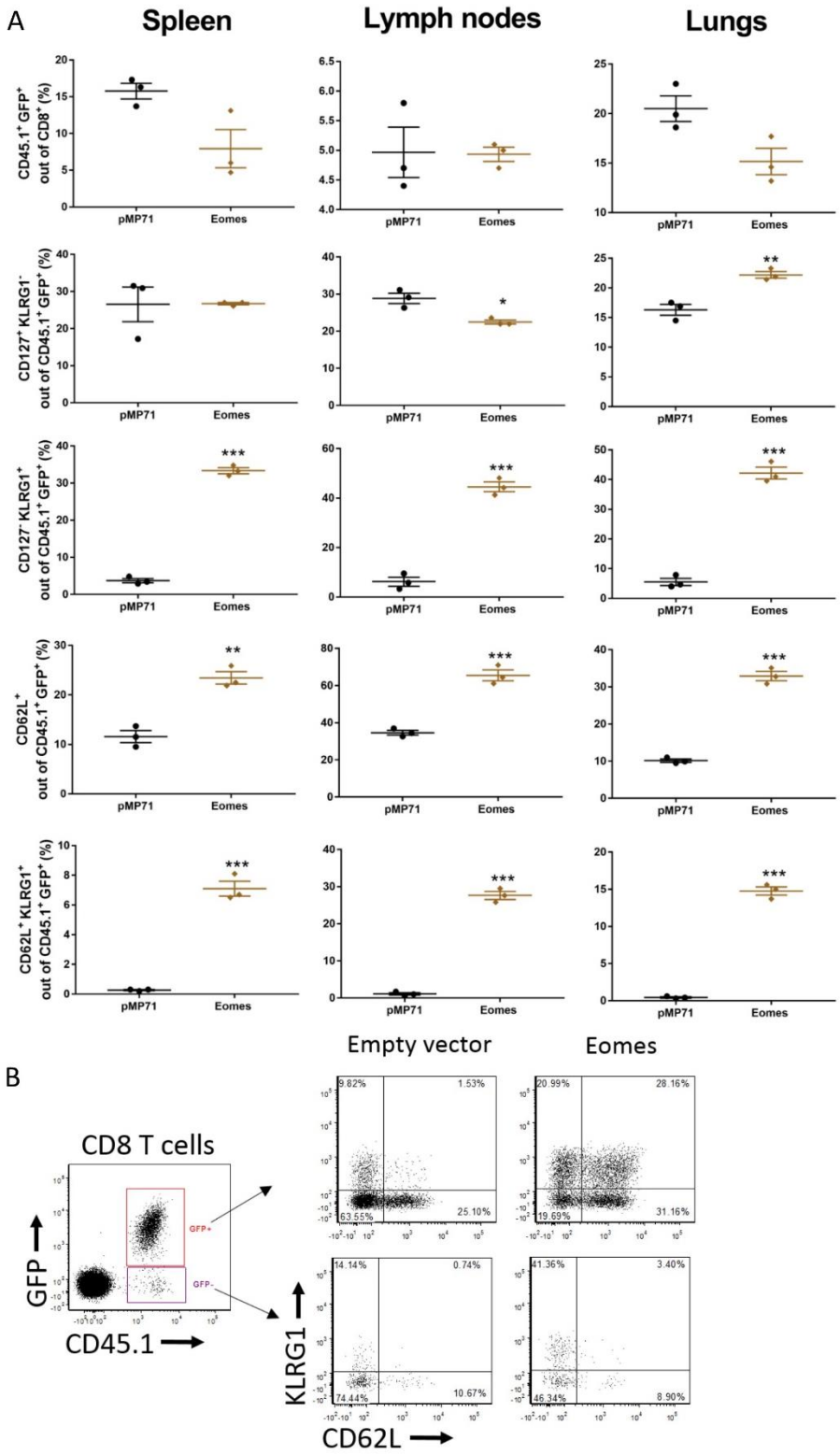

Figure 4.12: Differentiation and localisation of CD8 T cells overexpressing Eomes during a secondary challenge. Continuing from the experiment established in **Figure 4.11**. On day seven post vaccine rechallenge, the spleen, lymph nodes, and lung of mice were harvested from mice (**Supplemental figure 4.2**). A and B, the phenotype of transferred cells was assessed by flow cytometry. This experiment was performed once with three mice per group.

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4.3 Discussion

Retrospective analysis of clinical trials and preclinical murine models have reported that less differentiated CD8 T cell phenotypes provide more effective ACT therapy in part due to enhanced survival of transferred cells [290, 325, 368, 369, 380]. As such, identifying ways of generating large numbers of tumour-specific T cells that bear a memory phenotype but are still capable of effective tumour control has received considerable interest recently [381-383]. Here, I demonstrate that the overexpression of FOXO1, FOXO3a, or Eomes alters phenotypic aspects associated with memory differentiation without impacting the number of cells generated during *in vitro* expansion. Unfortunately, these modifications were ultimately deemed unsuitable for translation into ACT therapy based on the poor *in vivo* responses in vaccination or tumours models.

In a recent study by Restifo and colleagues, a pharmacological inhibitor of Akt was used during the *in vitro* expansion of CD8 T cells to improve the proportion of cells expressing a memory-like phenotype without compromising *in vitro* expansion [290]. Importantly, in their murine ACT model Akti-treated cells exhibited improved *in vivo* expansion upon repeated vaccination and provided greater anti-tumour immunity [290]. Although this illustrates a potential application of Akt manipulation, the downstream mechanisms of Akt-coordinated differentiation are incompletely understood, and further knowledge in this area may allow for precise fate coordination. Many studies have focused on FOXO transcription factors, direct downstream targets of Akt, as regulators of CD8 T cell migration and homeostatic proliferation [76-79]. For example, various knockout models have demonstrated that FOXO1 expression is required for the development of functional memory CD8 T cells [55, 79, 81, 82]. FOXO3a has also been linked to the promotion of CD62L expression [371], although within infection models it has also been shown to limit T cell survival due to its ability to promote the expression of pro-apoptotic factors such as BIM and PUMA [88]. Here, I questioned whether introducing CA FOXO1 or CA FOX3a had the potential to increase T_{cm} formation and, if this was the case, whether this would translate into improved efficacy for ACT [369].

In CD8 T cells, IL-2 stimulation diminishes CD62L expression, in part due to increased Akt activation reducing nuclear FOXO levels and causing the downregulation of KLF2 [78, 377]. The introduction of CA FOXO1 or CA FOXO3a into T cells enabled them to resist a substantial amount of CD62L downregulation in the IL-2 culture, indicating lower levels of effector differentiation, without impacting the expansion of cells *in vitro*. As FOXO3a has been shown to increase levels of

apoptosis via BIM and PUMA upregulation, the expansion of cells being unaffected was a surprising finding [91, 92, 374]. This does not rule out the possibility that greater levels of apoptosis were being compensated by enhanced proliferation and could be investigated in future studies. Achieving a high number of cells with a reduced terminal differentiated status was a promising start to this investigation yet the impact on survival, differentiation, and function required assessment in an *in vivo* setting to validate.

Within a non-lymphopenic environment CA FOXO1 did not enhance CD8 T cell engraftment or early memory recall in response to strong vaccination. This result was surprising as the memory-like profile promoted by CA FOXO1 normally favours *in vivo* survival and recall response compared to effector phenotypes [384]. The presence of CA FOXO3a was shown to reduce secondary expansion of CD8 T cells, which may have been a result of the aforementioned increased BIM and PUMA-mediated apoptosis [91, 92]. As these data were obtained from peripheral blood samples, the representation of the transduced population may have been affected by differential migration, with the CA FOXO favouring CD8 T cell homing to lymphoid organs, as shown in published findings for CA FOXO1 in T_{reg} cells [375]. However, while CA FOXO1 increased the frequency of CD8 T cells with a memory-like phenotype ($CD62L^{+}$ $KLRG1^{-}$) and significantly decreased the total number of transferred cells residing in the non-lymphoid organs, its presence did not increase the accumulation of transduced cells in the lymph nodes. Interestingly, a change in model revealed that CA FOXO1 favours engraftment of CD8 T cells within a lymphopenic environment, likely due to the increase in CD127 expression. This has the potential to improve LIP through enhanced BCL-2 mediated survival following increased IL-7 signalling, which would have been facilitated to a much higher degree in lymphopenic conditions with reduced competition for homeostatic cytokines [385].

The pharmacological inhibition of Akt in CD8 T cells during *ex vivo* expansion has been shown to improve anti-tumour immunity, attributed to the retention of a memory-like phenotype [290]. I was therefore interested in elucidating whether CA FOXO1 in CD8 T cells would generate similar benefits in preclinical models of ACT. As Akt inhibition in previous studies was limited to the *in vitro* culture of CD8 T cells, the retroviral transduction system offered a permanent modification of cells, even following the *in vivo* transfer. However, the ACT models explored in this chapter demonstrate that the presence of CA FOXO1 in CD8 T cells made anti-tumour immunity significantly worse. There are several possible explanations for this result. Firstly, although the presence of CA FOXO1 improved the frequency of transferred CD8 T cells upon peptide vaccination, these cells expressed lower levels of granzyme B, which might have limited their

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killing ability *in vivo*. Alternatively, It is also possible that CA FOXO1 limited peripheral migration due to the increased expression of lymphoid organ homing markers, thereby reducing the level of surveillance in the tumour environment [386]. This last point is supported by my early results, showing that CA FOXO1 limited accumulation in non-lymphoid organs, and also by published work demonstrating that CA FOXO1 expression in T_{reg} cells impedes the ability of cells to migrate to peripheral sites [375]. A possible solution to this constraint would be a retroviral plasmid with inducible expression, so that FOXO1 could be overexpressed during the *in vitro* culture, to generate cells with a memory like profile, but could be modulated to lower levels or completely lifted upon adoptive transfer. This may allow for plasticity of CD8 T cells, allowing them to regain effector function and migration as seen for the experiments utilising Akti during the *in vitro* culture to improve efficacy in preclinical ACT models [290].

Although the loss of FOXO1 severely limits memory generation [81, 82], the data here indicate that the constitutive activation of FOXO1 does not improve the functionality of CD8 T cells in regards to tumour control. Numerous signalling pathways triggered by signals 1, 2, and 3 converge on Akt activation, but also influence many other interrelated signalling pathways that are pivotal to CD8 T cell fate determination, ultimately regulating critical transcription factors such as T-bet, Eomes, Blimp, BCL-2, ID2, and ID3 [66]. Even within the PI3K/Akt pathway, factors other than FOXO1 are critical determinants of CD8 T cell differentiation, such as Wnt and Bach2 [387, 388]. It is therefore possible that overexpressing a single factor is not sufficient to guide differentiation into a functional memory cell while retaining high levels of effector function required for ACT. It is also possible that the *ex vivo* culture performed here was not sufficient to generate the levels of terminal differentiation that would be required for CA FOXO1 to provide a benefit to the cells' survival. Taken together the results here indicate that it is not of interest to introduce CA FOXO1 into CD8 T cells with the aim of optimising the ACT treatment against cancer. However, these data offer a novel insight into the role of FOXO1 in CD8 T cells with its constitutive activation enhancing a memory like profile while limiting peripheral migration and reducing the expression of effector molecules without enhancing the expansion capacity of memory cells upon vaccine restimulation.

Following these findings, I considered an alternative way of promoting the T_{cm} phenotype. FOXO1 has been shown to influence the T-bet:Eomes axis, which regulates CD8 T cell differentiation and function [45, 51]. The knockout of Eomes has been detailed to inhibit memory formation but also implicated in reducing cytokine expression [58]. Following *in vitro* culture in IL-7, the

overexpression of Eomes has been shown to improve the accumulation of T cells in response to peptide stimulation *in vitro* and *in vivo* [59]. As such, I investigated what impact overexpressing Eomes would have on differentiation and function of CD8 T cells in a translatable ACT protocol.

Transduced OT-I T cells overexpressing Eomes shared certain similarities with the introduction of CA FOXO1 as CD8 T cells resisted differentiation into the classical effector profile when expanded in IL-2, such as maintaining high levels of CD62L and lower levels of T-bet. Although there was downregulation of IL-2rα there was no difference in the extent of IL-2 mediated proliferation. However, it is possible that reduced IL-2 signalling is, in part, responsible for the reduced effector differentiation profile. Although these cells initially appeared to resist terminal differentiation, the *in vivo* vaccination of memory T cells over-expressing Eomes showed dramatically reduced ability to express IL-2 at the peak of a vaccine response, going against the established dogma for the T_{cm} phenotype [389]. Additionally, high levels of KLRG1 expression occurred for these cells regardless of whether the CD8 T cells expressed CD62L. Although the expression of KLRG1 is classically linked with effector function and terminal differentiation of CD8 T cells [390], this phenomenon did not influence the extent of the contraction phase for the cells overexpressing of Eomes. Again, mirroring the finding shown earlier for CD8 T cells expressing CA FOXO1, the upregulation of CD62L shown in cells overexpressing Eomes was not accompanied by enhanced localisation to lymphoid organs. With reduced IL-2 production and increased KLRG1 expression, the supraphysiological levels of Eomes appeared to promote aspects of a dysfunctional T cell phenotype similar to the exhaustion state seen in chronic infections. Supporting this theory are the findings from Wherry's group, who have indicated that high levels of Eomes can in fact be used as a marker to distinguish exhausted T cells subsets with reduced functionality [391]. Indeed, while T-bet has been classically described as a marker for terminal differentiation in the context of a chronic infection, T-bet^{hi} Eomes^{lo} cells are still able to proliferate in response to antigen and give rise to T-bet^{hi} Eomes^{hi} progeny that are terminally differentiated. It therefore seems likely that low or medium levels of Eomes expression are required for cells to transition into functional memory, whereas high or sustained levels of Eomes following stimulation can shut down aspects of effector T cells function. Data presented in this chapter indicate the over-representation of Eomes results in reduced T-bet levels, causes an imbalance in T cell differentiation, and hinders the ability of CD8 T cells to produce cytokines. Taken together, these findings indicate that the overexpression of Eomes is not appropriate for the production of T cells desirable for ACT.

As mentioned previously, higher levels of terminally differentiated CD8 T cells have been correlated with reduced *in vivo* persistence and thus reduced the efficacy of ACT therapy against

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cancer. However, effector cells may be very important for initiating an anti-tumour response due to their ability to patrol peripheral sites and instigate immediate cytotoxic responses. Constitutively promoting factors associated with memory formation may therefore be an ineffective approach to optimise ACT. This is supported by the finding here that neither overexpressing Eomes nor CA FOXO1 could improve memory CD8 T cell responses to *in vivo* vaccination despite both factors being crucial in the development of functional memory cells. Indeed, similar to the finding shown here for CA FOXO1, a previous study has shown that overexpression of proline-rich Akt substrate of 40 kDa (PRAS40), a negative regulator of mTOR, promoted a memory phenotype but T cells were unable to mediate anti-tumour immunity [392]. This system highlighted the importance of functional effector cells within the population for the initiation of tumour destruction. Potentially future work could evaluate inducible genetic factors or transient use of pharmacological molecules to promote memory formation during the *ex vivo* culture thereby limiting excessive levels of terminal differentiation. This may be of benefit to ACT for the expansion of large numbers of non-terminally differentiated T cells that retain the transcriptional plasticity required to gain effector function and destroy tumour cells upon *in vivo* transfer. While exploring the manipulation of FOXO1, FOXO3a, and Eomes has offered novel insights into these transcription factors, their maintained overexpression in CD8 T cells is inappropriate for augmenting ACT efficacy against cancer.

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Chapter 5: Manipulating T cell sensitivity for enhancing ACT efficacy

5.1 Introduction

Within the tumour microenvironment numerous factors can inhibit the stimulation of T cells thereby suppressing the anti-tumour response. As discussed in **Chapter 1.3**, these factors include the presence of T_{reg} cells, MDSC, checkpoint molecules, and anti-inflammatory cytokines that restrain the activation, proliferation, and effector function of T cells [224, 393, 394]. Under normal circumstances mechanisms of T cell suppression are in place to ensure adequate tolerance towards self-antigen and attenuate the risk of an autoimmune response. However their overrepresentation in tumours allows cancer cells to escape immune-mediated destruction. Simply blocking or removing inhibitory factors can be sufficient to lift suppression and lead to a robust anti-tumour T cell response. This has been illustrated by the success of mAbs targeting inhibitory checkpoint receptors, PD-1 and CTLA-4, for the treatment of advanced non-small-cell lung cancer, melanoma, renal-cell cancer, and ovarian cancer [395-398]. In the field of ACT therapy against cancer, the ability to genetically modify T cells prior to transfer offers a platform for enhancing their functionality. Directly manipulating intracellular signalling pathways that regulate T cell activation or survival can provide permanent alterations to effector function or longevity, thereby giving the transferred cells a better chance of overcoming suboptimal stimulation and improving the therapies efficacy. For example, preclinical work has shown that CRISPR disruption of PD-1 in T cells grants improved anti-tumour responses within murine ACT models [344]. Interestingly, this approach was validated alongside the lentiviral incorporation of a CAR, demonstrating the feasibility of multiplex editing to generate tumour specific T cells that have reduced susceptibility to suppression [344]. Within this area of research there are many novel targets that have not been fully explored and require validation. Here I focus on augmenting T cell stimulation through the knockdown of several PTPs (PTPN2, PTPN7, and PTPN22; see **Chapter 1.2.10** for background information) and a tyrosine kinase (CSK; See **Chapter 1.2.11** for background information), the known targets for which are summarised in **Table 5.1**.

	Targets	**Reported role**
PTPN2	JAK1 [121], JAK3 [117], Lck [124] , Fyn [124].	Reduces naïve T cell sensitivity towards low-affinity TRC interactions and cytokine stimulation.

PTPN7	ERK2 [131].	Possibly involved in negative feedback from T cell stimulation.
PTPN22	Lck, Fyn, Zap70 [97, 116, 139, 142]	Reduces naïve T cell sensitivity towards low-affinity TCR interactions.
CSK	Lck [155].	Reduces naïve T cell sensitivity towards low-affinity antigens.

Table 5.1: Major known targets and roles for PTPN2, PTPN7, PTPN22, and CSK in CD8 T cells. Although these proteins have many unique targets they have all been reported limiting aspects of the signalling cascade following TCR stimulation.

5.1.1 Project aims

The focus of this work was to test if shRNA mediated knockdowns of PTPN2, PTPN7, PTPN22, and CSK could augment the function of activated CD8 T cells for improved ACT efficacy. This entailed (i) delineating the functional consequence of these knockdowns in OT-I T cells through *in vitro* screening of differentiation, cytokine production, and ability to kill target cells, (ii) testing *in vivo* responsiveness to vaccination and (iii) performance within an ACT therapy against EG7 tumours. It is worth noting that the adoptive transfer model used for this section was particularly rigorous, without the sub-lethal irradiation preconditioning to generate lymphopenia or the use of vaccination.

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5.2 Results

Firstly, the retroviral shRNA vectors targeting the phosphatases PTPN2, PTPN7, and PTPN22 (constructed in **Chapter 3.2.3**) were validated for their ability to mediate the knockdown of their target protein. Four shRNA sequences (A,B,C, and D) were used to target PTPN7 and PTPN22, while just one previously published shRNA sequence for PTPN2 knockdown was used [113] (See **Chapter 2.1.7** for sequences). To assess the protein knockdown in the context of the preclinical model established in **Chapter 3.2.1**, OT-I splenocytes were activated, transduced, purified by FACS (GFP^+), and cultured in IL-2 for five days to facilitate the expansion of effector CD8 T cells. Following this expansion, the cells were lysed to obtain total protein, from which the relative levels of PTPN2, PTPN7, or PTPN22 were determined by western blot (**Figure 5.1A**). While the different constructs varied in their ability to mediate protein knockdown, results here demonstrate the most successful shRNA sequence from each group could consistently reduce the expression level of the target phosphatase by more than 75% relative to CD8 T cells transduced with the shScrambled control vector (**Figure 5.1B**). On average, the shRNA construct targeting PTPN2 (shPTPN2) achieved an 82% knockdown. The shPTPN7 D construct (herein referred to as shPTPN7) produced the best results within its cohort, averaging a 77% knockdown, while the shPTPN22 B construct (herein referred to as shPTPN22) produced the best results within its group, averaging an 86% knockdown. As such, these data confirmed that each shRNA substantially reduced the target phosphatase, between four and five-fold knockdown, in the context of IL-2 expanded CD8 T cells. The shRNA vectors were then sequenced, confirming the shRNA sequences were correctly incorporated into the constructs (**Supplementary figure 5.1**).

STAT5, a known substrate of PTPN2, is downstream of IL-2 and IL-15 signalling. It was therefore of interest to assess whether the knockdown of PTPN2 or the other phosphatases, would impact the differentiation of cells during cytokine-mediated expansion. Following the standard transduction of OT-I splenocytes with the shPTPN2, shPTPN7, shPTPN22, or shScrambled retrovirus, the GFP^+ transduced cells were purified by FACS and expanded *in vitro* in IL-2 or IL-15 to achieve effector-like and memory-like profiles respectively [399]. For the OT-I T cells transduced with the control vector classical differentiation profiles were achieved, with activation and effector markers expressed at high levels for IL-2 expanded CTLs and expressed at much lower levels by the cells expanded in IL-15 (**Figure 5.1C**). Whether the cells were cultured in IL-2 or IL-15, the results here show that the presence of PTPN2, PTPN7, or PTPN22 knockdown made no visible impact on the phenotypic differentiation of CD8 T cells according to markers of memory (CD62L, CD127, and

Eomes), activation (CD25, CD69, and T-bet), or effector function (granzyme B) (**Figure 5.1C** and **Figure 5.2**).

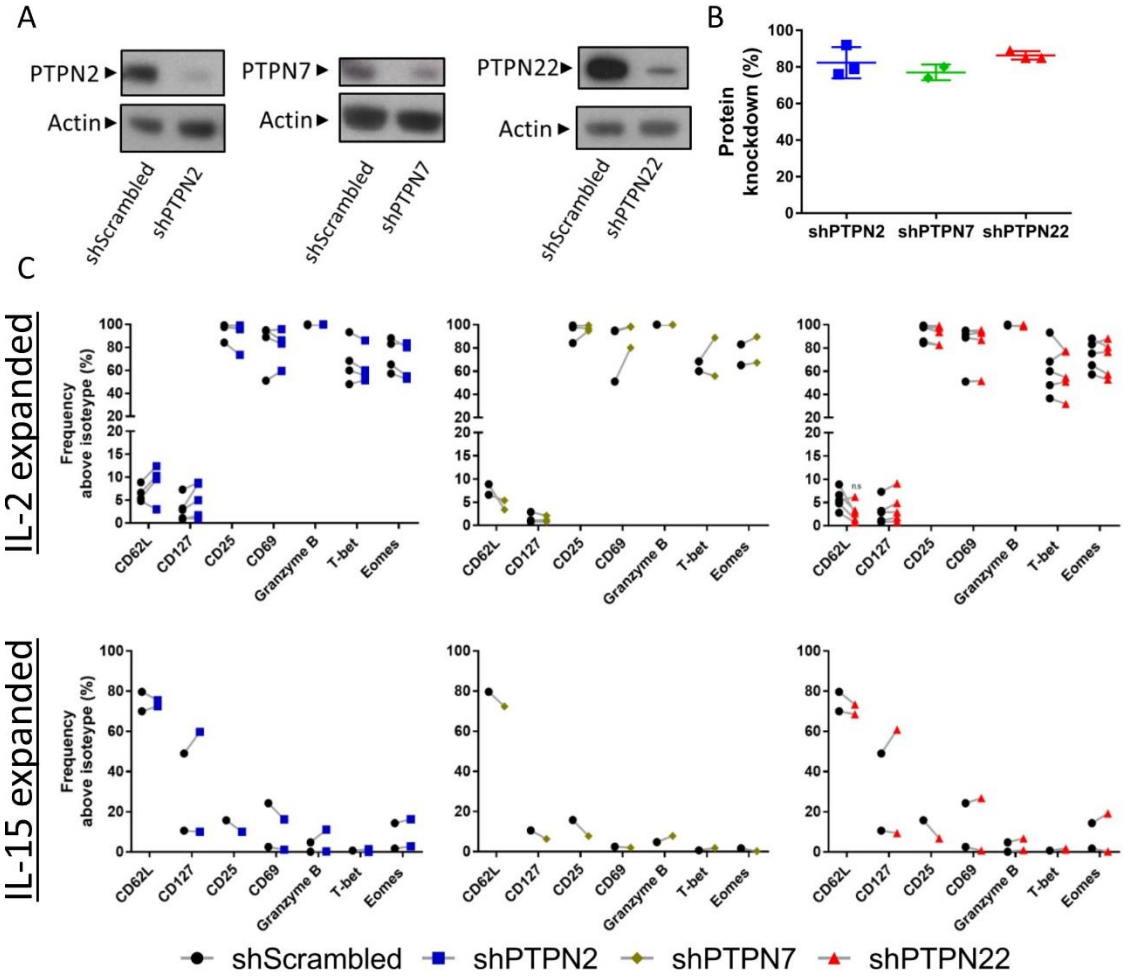

Figure 5.1: The shRNA knockdown of phosphatases and their effect on cytokine-mediated differentiation *in vitro*. Naïve OT-I splenocytes were activated and transduced with shPTPN2, shPTPN7, shPTPN22 or shScrambled following the standard protocol. A, The transduced cells were purified by FACS (GFP^+) and expanded for five days in IL-2 (10ng/ml) refreshing cytokine daily. The cell population were lysed and protein expression for PTPN2, PTPN7, PTPN22, and Actin were assessed by western blot. B, The protein knockdown was quantified by densitometry from the western blot images, with the knockdown levels shown relative to the scrambled control, each being standardised against actin levels. These data are pooled from 2 or 3 independent experiments. C, Following FACS purification, the transduced OT-I were culled in either IL-2 (10ng/ml) or IL-15 (15ng/ml) for 5 days, with cytokines refreshed daily. Following the expansion, the cell phenotype was assessed by flow cytometry. These data are pooled from 1 to 6 independent experiments (representative examples are shown in **Figure 5.2**).

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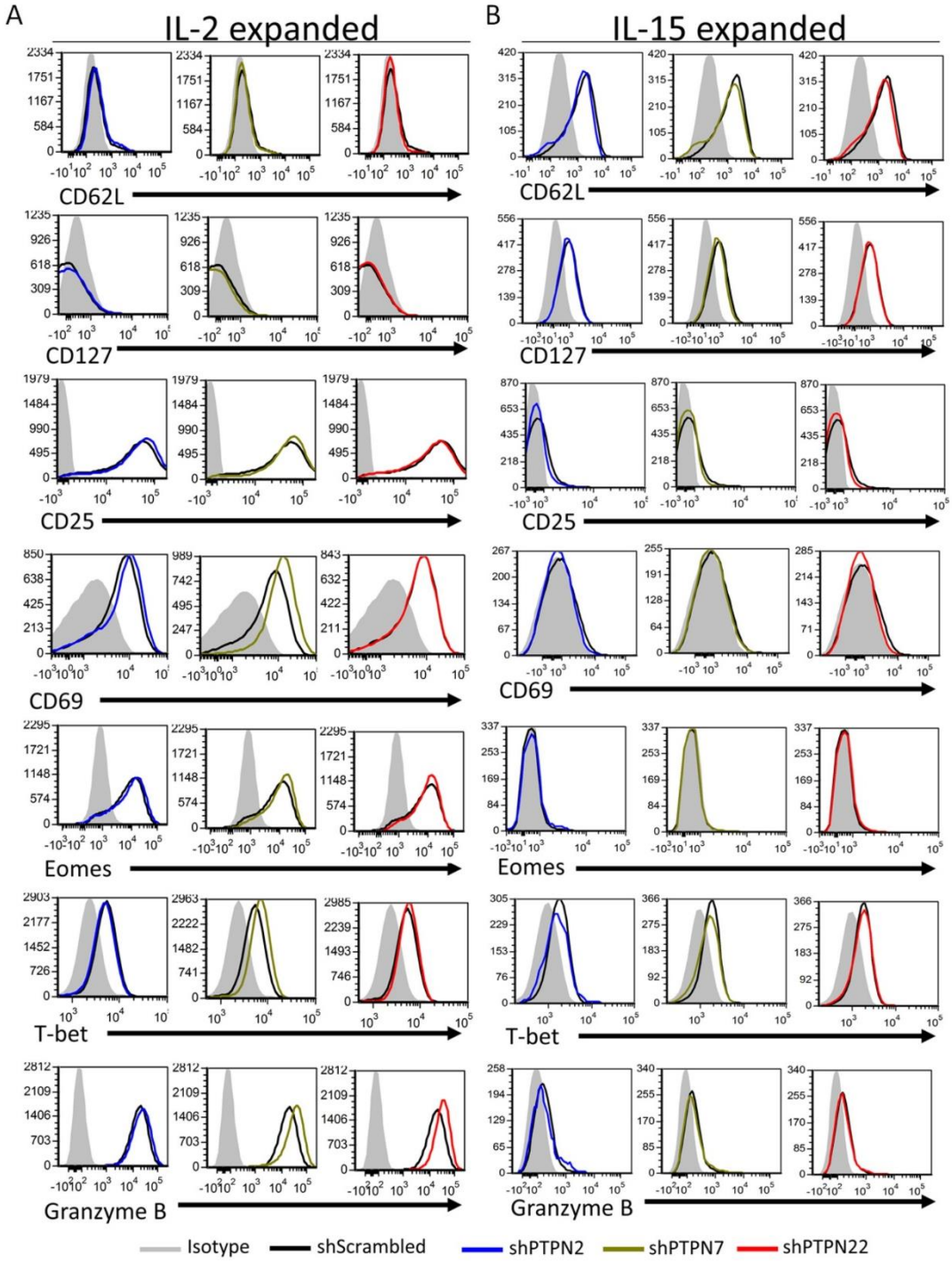

Figure 5.2: The effect of phosphatase knockdown on the cytokine-mediated differentiation of CD8 T cells *in vitro*. Representative histograms of the data presented in **Figure 5.3C**.

It was next of interest to determine if the knockdown of the phosphatases impacted the capacity of OT-I cells to produce cytokines following TCR stimulation. These experiments aimed to establish if the described role of PTPN7 in suppressing NFAT activation [130] could affect CD8 T cells sensitivity towards TCR signalling, as current understanding within this area is still limited.

Additionally, PTPN2 and PTPN22 have both been shown to limit the activation of naïve T cells following the TCR interactions [116, 127]. However, the impact of inhibiting these phosphatases in activated T cells that have been expanded in IL-2 in preparation for ACT is currently unknown. To fully assess whether the sensitivity to peptide stimulation was altered by the presence of these phosphatase knockdowns, peptides covering a range of affinities for the OT-I TCR were used (SIIN>SIIQ>SIIT) each titrated over a broad range of concentrations (10^{-5} to 10^{-9} M). The FACS purified OT-I CD8 T cells, transduced with shPTPN2, shPTPN7, shPTPN22, or shScrambled retrovirus, were expanded for 5 days in IL-2 before being restimulated with CD8 depleted irradiated feeder cells for four hours in the presence of peptide to assess the production of IL-2, IFNγ, and TNFα. Interestingly, both PTPN2 and PTPN22 knockdowns had very similar effects, significantly increasing the percentage of cells expressing IL-2 in response to peptide regardless of affinity (**Figure 5.3A and Figure 5.4**). The difference in IL-2 expression was particularly obvious at the high peptide concentrations and no additional benefit to the fold change in IL-2 expression observed with the use of low concentrations of peptide. While the difference in IL-2 production was striking, PTPN2 or PTPN22 knockdowns had no impact the expression of either IFNγ or TNFα (**Figure 5.3A and Supplementary figure 5.2**). Furthermore, the results here demonstrated that the knockdown of PTPN7 did not influence the expression of IL-2, IFNγ, or TNFα upon peptide stimulation (**Figure 5.3B**). As the PTPN2 and PTPN22 knockdown had a clear impact on the functionality of the CD8 T cell following TCR stimulation, these modifications were taken forward for further testing.

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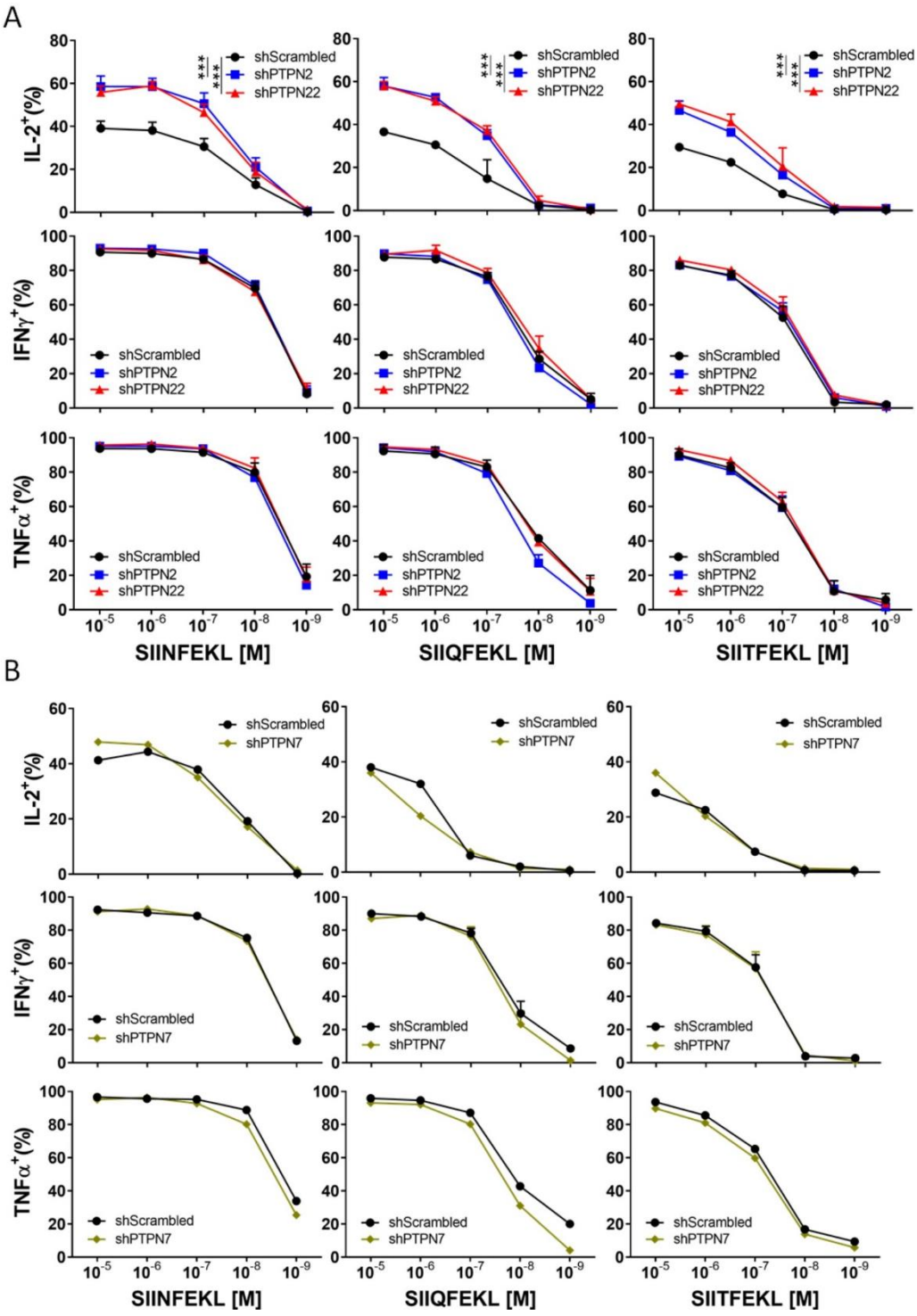

Figure 5.3: The effect of phosphatase knockdown on cytokine production by CTLs. A and B, Naïve OT-I splenocytes were activated and transduced with shPTPN2, shPTPN7, shPTPN22, or shScrambled retrovirus following the standard protocol. The transduced CD8 T cells were purified by FACS (GFP^+ $CD4^-$) and expanded for five days in IL-2 (10ng/ml) refreshing cytokine daily. Following the expansion, the transduced cells were cultured for four hours with CD8 depleted irradiated splenocytes, Golgi-plug and the peptide indicated. The cytokine production was then assessed by flow cytometry. A, These data are pooled from 2-3 independent experiments (representative of 4-6 experiments), statistics are shown as 2 way ANOVA (Bonferroni). B, Data are pooled from 2 independent experiments

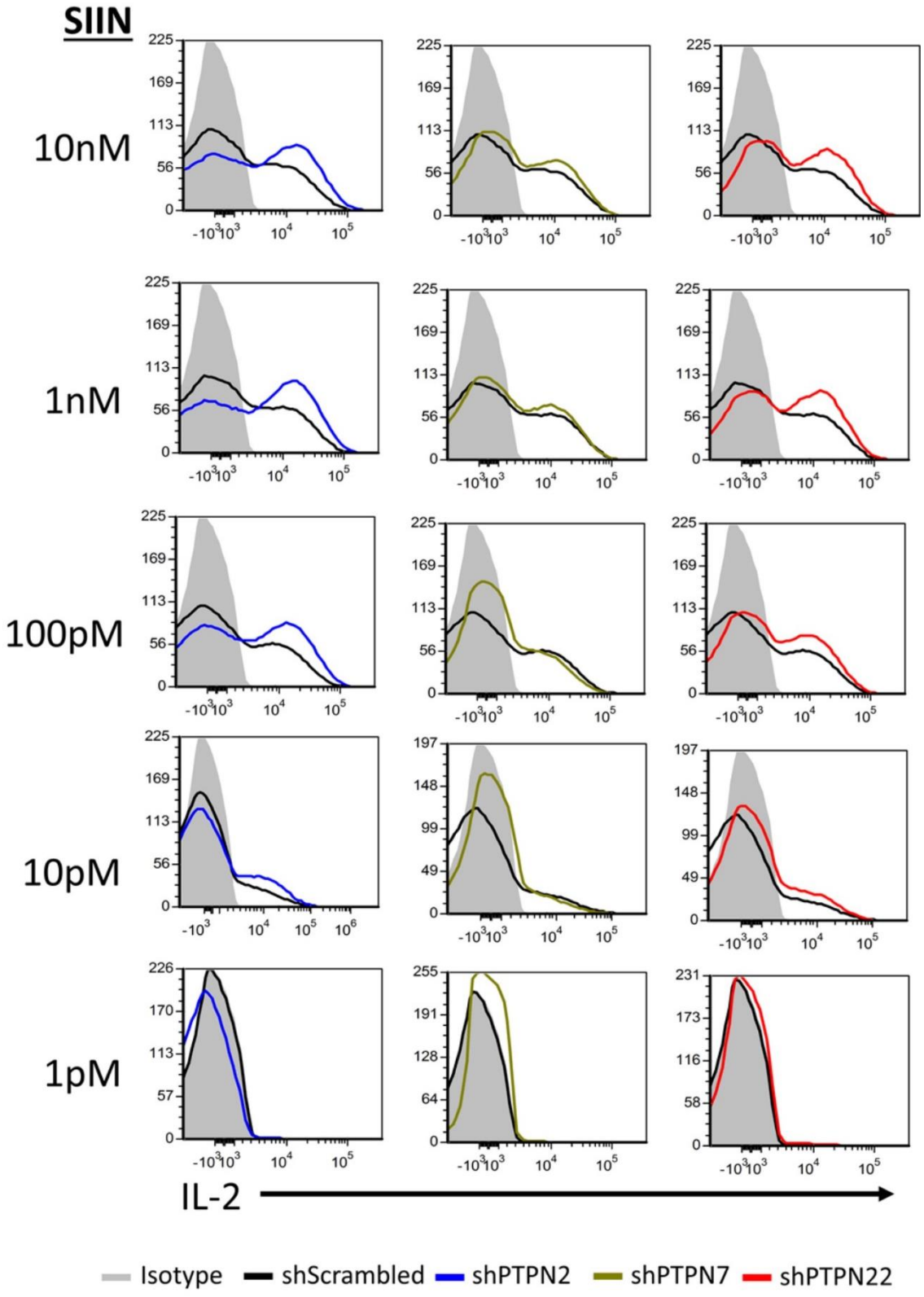

Figure 5.4: The effect of phosphatase knockdown on IL-2 production by CTLs. Representative histograms from the data described in **Figure 5.5 A and B**

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Since the knockdown of either PTPN2 or PTPN22 increased the sensitivity to TCR stimulation in terms of IL-2 expression, but not IFNγ or TNFα expression, I questioned whether this was a result of transcriptional or translational differences either before or after TCR stimulation. To address this, transduced and purified OT-I CD8 T cells expressing shScrambled, shPTPN2, or shPTPN22, expanded them in IL-2 for six days and restimulated the cells for 4 hours alongside irradiated splenocytes, comparing a high concentration of peptide (SIIN 10nM) stimulation to non-restimulated controls. Cytokines production profile was confirmed by FACS and mRNA was isolated from MACS-purified CD45.1^{+} transduced cells, attaining 90% purity for the non-restimulated groups and 70% purity for the non-restimulated groups (**Supplemental Figure 5.3A**). As the purity was consistent within these two groups, this allows for a valid comparison between the different knockdowns to be made. The results here indicate that the baseline IL-2 mRNA in non-restimulated cells was extremely low, on the limit of the qPCR detection threshold approximately 19 cycles after the housekeeping gene B2M [400] (**Supplemental Figure 5.3B**). Therefore, the significant (1.7 fold) increase in IL-2 mRNA observed in the non-restimulated cells of the shPTPN22 group may not actually reflect a substantial increase in IL-2 protein (**Figure 5.5A and B**). Following restimulation, there was an upregulation of IL-2 mRNA, with the PTPN2 or PTPN22 knockdowns mediating a significant (approximately 1.8-fold) increase above the levels seen in the control group (**Figure 5.5A**). Interestingly, for the non-restimulated CD8 T cells the IFNγ mRNA was significantly elevated by the knockdown of PTPN2 (1.5-fold) and PTPN22 (1.4-fold) (**Figure 5.5A**). Although there was no IFNγ expression at the protein level for non-stimulated cells, its mRNA was reliably detectable (**Supplementary Figure 5.3B**). Upon restimulation, IFNγ mRNA in the cells possessing PTPN2 and PTPN22 knockdowns was marginally, yet significantly, elevated (**Figure 5.5A).** Finally, the levels of TNFα mRNA was not altered by the PTPN2 or PTPN22 knockdown in non-stimulated cells. However, upon restimulation, TNFα mRNA was significantly enhanced by the knockdown of PTPN2 (1.5-fold) and PTPN22 (1.4-fold) (**Figure 5.5A**). It is interesting that this change in mRNA had no impact on the TNFα expression observed at the protein levels.

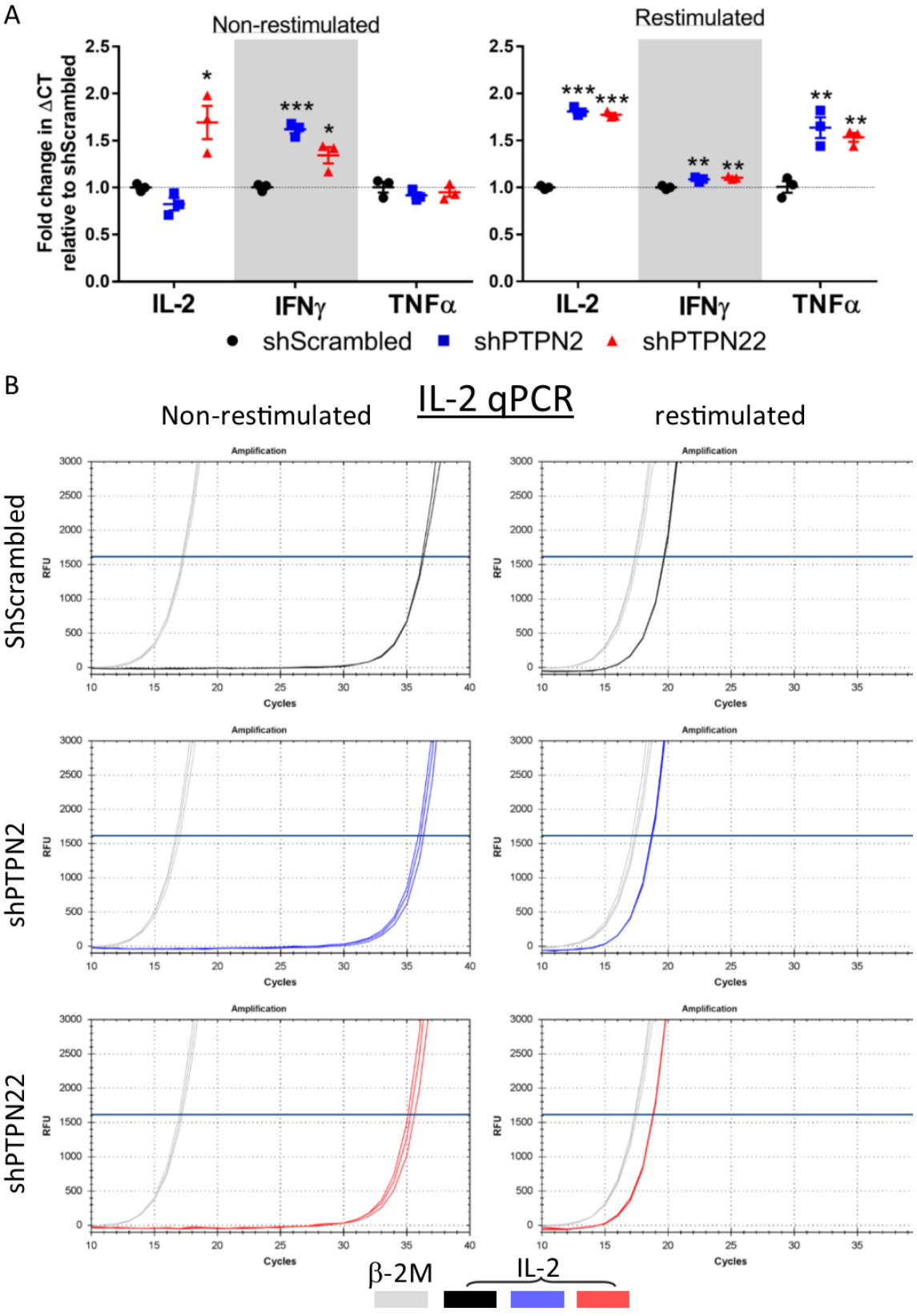

Figure 5.5: The effect of phosphatase knockdown on cytokine mRNA expression by CTLs. A and B, Naïve OT-I splenocytes were activated and transduced with shPTPN2, shPTPN22, or shScrambled retrovirus following the standard protocol. The transduced cells were purified by FACS (GFP^+ $CD4^-$) and expanded for six days in IL-2 (10ng/ml) refreshing cytokine daily.

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Following the expansion, the transduced cells were cultured for four hours with CD45.1^{-} CD8 depleted irradiated splenocytes alone or with SIIN (10nM) peptide. CD45.1^{+} transduced cells were then purified by MACS and RNA was isolated from the cell population. The mRNA levels of IL-2, IFNγ, TNFα, and B2M were assessed by qPCR. A, Fold change in ΔCT standardised to the control shScrambled group. Data shows three replicates from one experiment. B, Data from the amplification curves acquired from qPCR.

Although the effector phenotype, as measured by expression of granzyme B, IFNγ, and TNFα, was not influenced by the knockdown of PTPN2 or PTPN22 in IL-2 expanded CTLs, it was still important to assess whether these knockdowns influence the ability of CD8 T cells to kill target tumour cells. To explore this, shPTPN2, shPTPN22, or shScrambled transduced OT-I cells were cultured in IL-2 as before to generate effector T cells. CFSE -stained EG7 tumour cell targets were mixed with an equal number of CTV-stained EL4 (non-OVA expressing equivalent tumour) cells then co-culture with transduced T cells at various target ratios for four hours. The level of specific killing assessed by comparing the ratio of remaining EG7 to EL4 cells demonstrated that the knockdown of PTPN2 or PTPN22 did not influence the killing ability of IL-2 expanded OT-I T cells (**Figure 5.6 and Figure 5.7A**). Furthermore, these findings were recapitulated using SIIN pulsed EL4 cells as target cells (**Supplementary Figure 5.4**). These data highlighted that the inhibition of PTPN2 or PTPN22 confers no impact on the ability of CTLs to kill target tumour cells *in vitro* following IL-2 expansion.

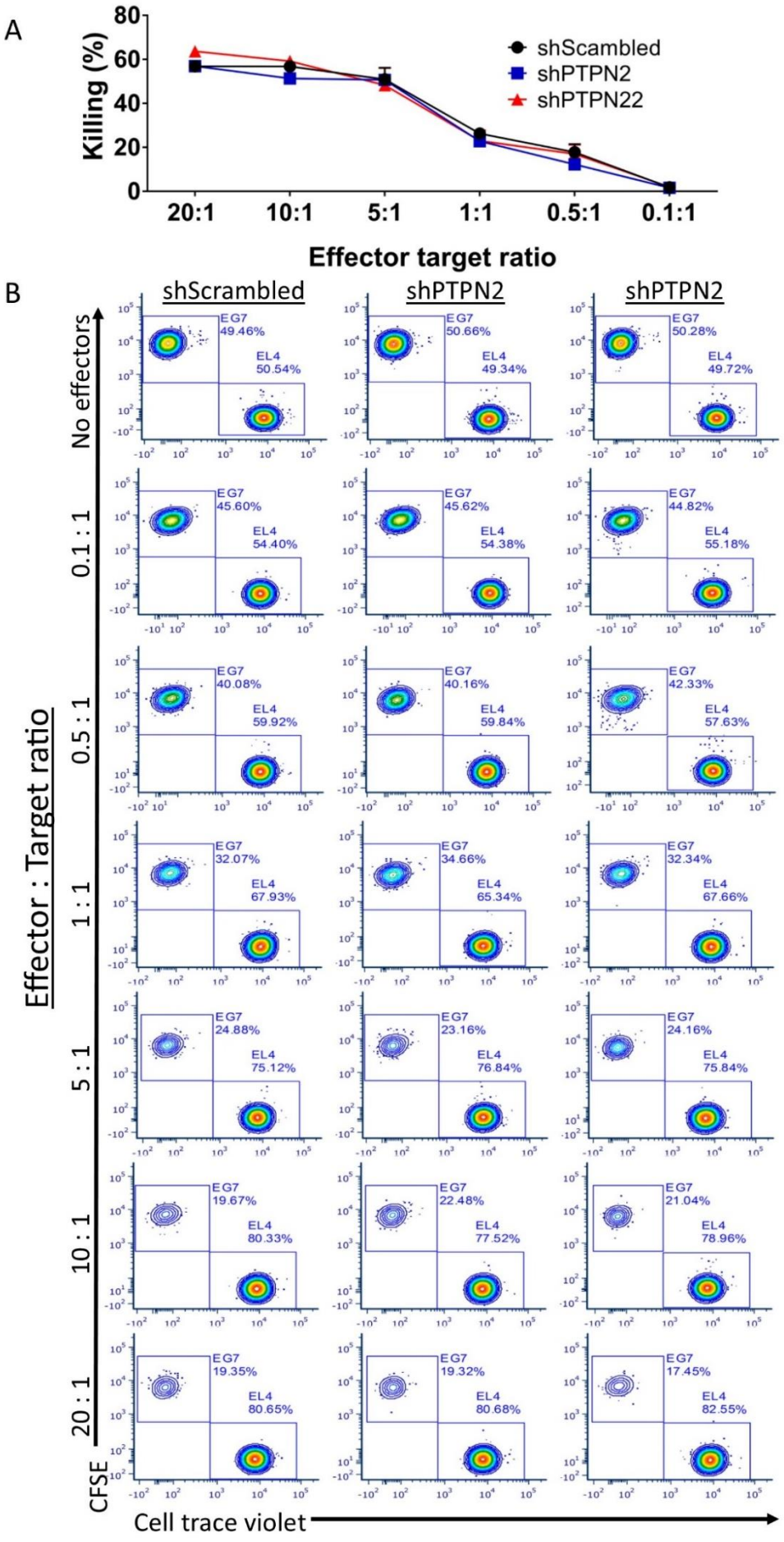
A
Killing (%)
80
60
40
20
0
shScambled
shPTPN2
shPTPN22
20:1
10:1
5:1
1:1
0.5:1
0.1:1
Effector target ratio
B
shScrambled
shPTPN2
shPTPN2
Effector : Target ratio
No effectors
0.1 : 1
0.5 : 1
1 : 1
5 : 1
10 : 1
20 : 1
EG7 49.46%
EL4 50.54%
EG7 50.66%
EL4 49.34%
EG7 50.28%
EL4 49.72%
EG7 45.60%
EL4 54.40%
EG7 45.62%
EL4 54.38%
EG7 44.82%
EL4 55.18%
EG7 40.08%
EL4 59.92%
EG7 40.16%
EL4 59.84%
EG7 42.33%
EL4 57.63%
EG7 32.07%
EL4 67.93%
EG7 34.66%
EL4 65.34%
EG7 32.34%
EL4 67.66%
EG7 24.88%
EL4 75.12%
EG7 23.16%
EL4 76.84%
EG7 24.16%
EL4 75.84%
EG7 19.67%
EL4 80.33%
EG7 22.48%
EL4 77.52%
EG7 21.04%
EL4 78.96%
EG7 19.35%
EL4 80.65%
EG7 19.32%
EL4 80.68%
EG7 17.45%
EL4 82.55%
CFSE
Cell trace violet

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Figure 5.6: The effect of phosphatase knockdown on the killing ability of CTLs. A and B, Naïve OT-I splenocytes were activated and transduced with shPTPN2, shPTPN22, or shScrambled following the standard protocol. Transduced cells were purified by FACS (GFP^+) and expanded for five days in IL-2 (10ng/ml) refreshing cytokine daily. Following the expansion, the transduced cells were cultured for four hours with a 1:1 mix of EG7 target cells stained in CFSE and EL4 cells non-target cells stained with CTV. Following this incubation, the ratio of EG7 to EL4 was determined by flow cytometry by gating on the $CD45.1^-$ population. A, the specific killing of EG7 cells shown in triplicate. B, Representative plots from the raw flow cytometry data. These data are representative of two independent experiments.

At this stage of the investigation another vector was developed for the knockdown of CSK. Similarly to PTPN2 and PTPN22, studies have shown that the disruption of CSK enhances Lck signalling, increasing the levels of CD8 T cell activation in response to TCR stimulation. It was therefore of interest to test if CSK knockdown can mediate a similar effect in the context of the transduced CTLs generated within the ACT protocol. Firstly, the ability of constructs, shCSK (A,B,C, and D) (built in **Chapter 3.2.3**), to mediate protein knockdown was assessed. Following the FACS purification (GFP^+), transduced cells were expanded in IL-2 for five days, before lysing the populations to acquire total protein. Here, the most successful shRNA sequence, shCSK C (herein referred to as shCSK), achieved an average 95% knockdown relative to the shScrambled group (**Figure 5.7A and B and Supplementary figure 5.5**). The shCSK vector was then sequenced, validating the correct insertion of the shRNA, although it was not possible to sequence the middle section of the shRNA construct despite multiple attempts to weaken the hairpin structure by modifying the reaction conditions (**Supplementary figure 5.6**). To determine if knockdown of CSK would affect the differentiation of CD8 T cells during cytokine-mediated expansion, purified transduced cells were cultured in IL-2 or IL-15 to generate effector-like or memory-like profiles respectively. Here it was shown that the CSK knockdown did not affect the differentiation of expanded in IL-2 or IL-15 as shown by the expression of memory (CD62L and CD127) and activation markers (CD25 and T-bet), and an effector molecule (granzyme B) (**Figure 5.7C**). Finally, the effect of CSK knockdown on the ability of OT-I T cells to produce cytokines following restimulation was assessed. Mirroring the results obtained from the PTPN2 and PTPN22 knockdowns, the CSK knockdown significantly increased IL-2 production at high concentrations of peptide (SIIN) (**Figure 5.7D**). This trend was maintained when the cells were stimulated with low-affinity peptides (SIIQ and SIIT). The expression of IFNγ and TNFα were not affected by the CSK knockdown. Taken together, the knockdown of CSK had a similar impact to the PTPN2 or PTPN22 knockdown on the phenotype of *in vitro* generated CTLs.

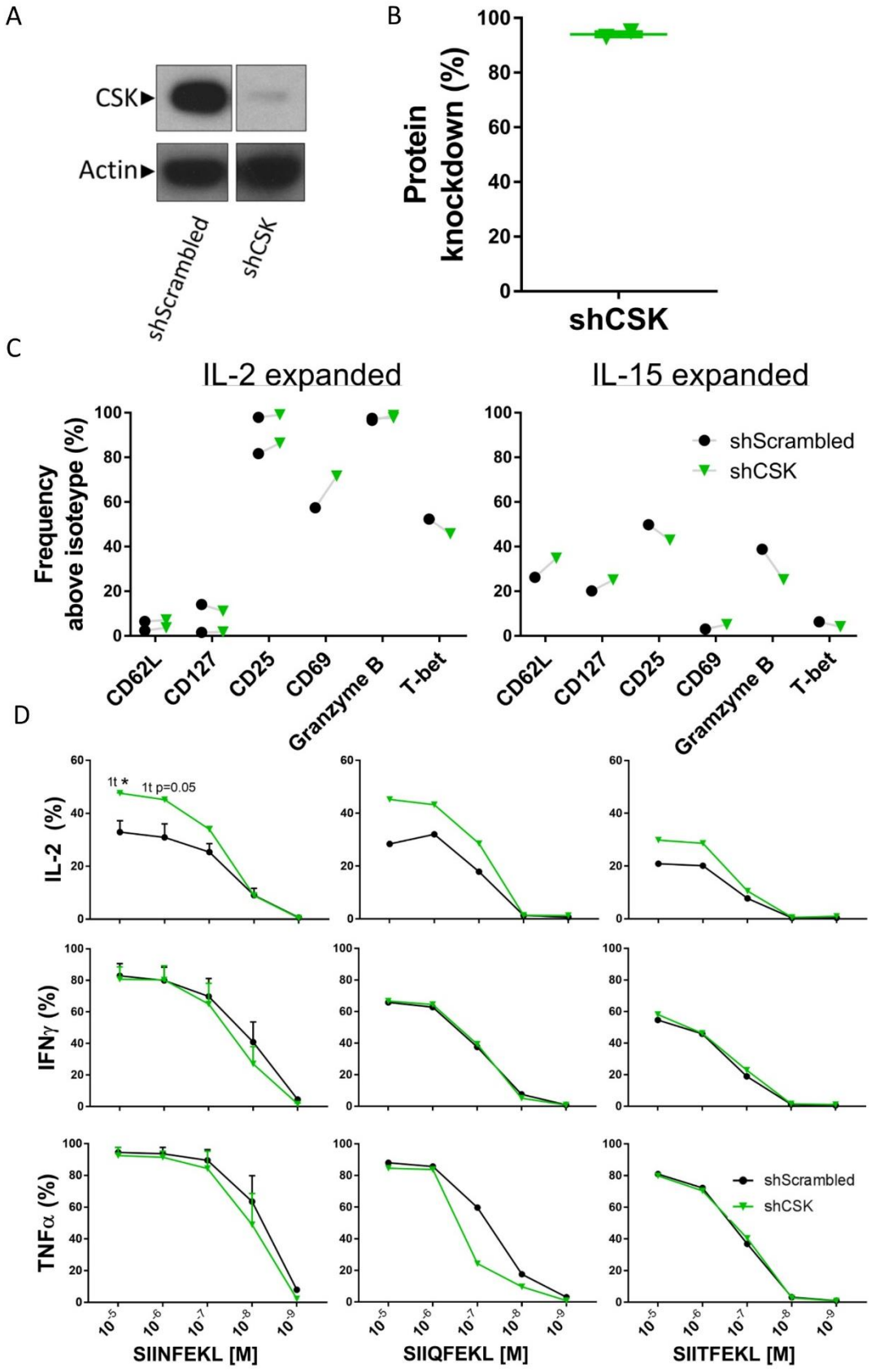

Figure 5.7: Validation of CSK shRNA knockdown and its effect on CD8 T cell phenotype and function *in vitro*. Naïve OT-I splenocytes were activated and transduced with retrovirus, shCSK

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or shScrambled, following the standard protocol. The transduced cells were purified by FACS (GFP^+) and expanded for five days in IL-2 (10ng/ml) or IL-15 (15ng/ml in the case of D, right panel) refreshing cytokine daily. A, IL-2 expanded cells were lysed, and CSK protein quantity was assessed by western blot. For the continuous western blot see **Supplementary figure 5.5** B, The protein knockdown was quantified by densitometry on the western blot images, with the knockdown levels relative to the scrambled control standardised against actin levels. C, Pooled phenotypic data as determined by flow cytometry post-expansion in IL-2 (Left panel) or IL-15 (right panel) for 5 days. E, Following IL-2 expansion, the transduced cells were cultured for four hours with CD8 depleted irradiated splenocytes, Golgi-plug and the peptide indicated. The cytokine production of transduced cells was then assessed by flow cytometry.

Next, it was important to assess whether the introduction of the PTPN2, PTPN22, or CSK knockdown would influence OT-I T cells response to *in vivo* stimulation. To investigate this, non-purified transduced populations of OT-I T cells were transferred into WT C57BL/6, which then received a peptide vaccination (30nM SIIN) alone (**Figure 5.8A**) or with co-stimulation (50μg αCD40) (**Figure 5.8B**). The ratio of the transferred transduced (GFP^+) to non-transduced (GFP^-) $CD45.1^+$ cells was assessed over time from peripheral blood samples to determine the impact of the knockdowns. The results here demonstrated that the presence of PTPN2 knockdown significantly enhanced the proportion of GFP^+ cells six days after the peptide vaccination alone (granting a 1.9-fold increase) or with costimulation (granting a 1.5-fold increase) (**Figure 5.9A**). Interestingly, the presence of the PTPN2 knockdown had a positive bystander effect. This is shown as at the peak of the response there was a significant improvement for accumulation of total $CD45.1^+$ CD8 T cells in the shPTPN2 group for both peptide vaccination alone (8.4-fold increase) and with costimulation (4-fold increase), which cannot be fully accounted for by the increase in GFP^+ cells described early (**Figure 5.9B**). The knockdown of PTPN2 was also shown to improve the total number of GFP^+ cells at the peak of the response, normalised for differences in the initial GFP level, for both peptide vaccination alone (6-fold) or with costimulation (3-fold) compared to control group (**Figure 5.9C**). Interestingly, for the peptide vaccination alone, the PTPN2 knockdown gave significantly greater levels of effector differentiation, with approximately twice the frequency of $KLRG1^+$ $CD127^-$ cells at day 6 and 13 post-vaccination compared to the control group (**Figure 5.9D**). However, this effect was not observed in the costimulation vaccine setting. Unlike the knockdown of PTPN2, the presences of the PTPN22 knockdown had no influence on the accumulation or differentiation of the OT-I cells in either of the vaccination settings (**Figure 5.9A-C**). CSK knockdown affected the kinetics of the OT-I response, with the accumulation of cells peaking later than in the other groups, appearing closer to day eight post vaccination (compared to day six for the control group) in both settings. Overall, the knockdown of CSK was detrimental

to the accumulation of OT-I cells, as shown by a significant decrease in the GFP^+ cells at day 14 post-vaccination (**Figure 5.9A**). Interestingly, the presence of shCSK appeared to increase the proportion of CD8 T cells with an effector/ terminally differentiated phenotype on day 6 and eight post-vaccination in the co-stimulation setting (**Figure 5.9D**). Taken together, the results show that the PTPN2 knockdown offers intrinsic and extrinsic benefits to the CD8 T cells response to vaccination with more pronounced effects seen without costimulation. In contrast, the knockdown of PTPN22 had no impact on the response to vaccination and the knockdown of CSK was detrimental to the accumulation of cells.

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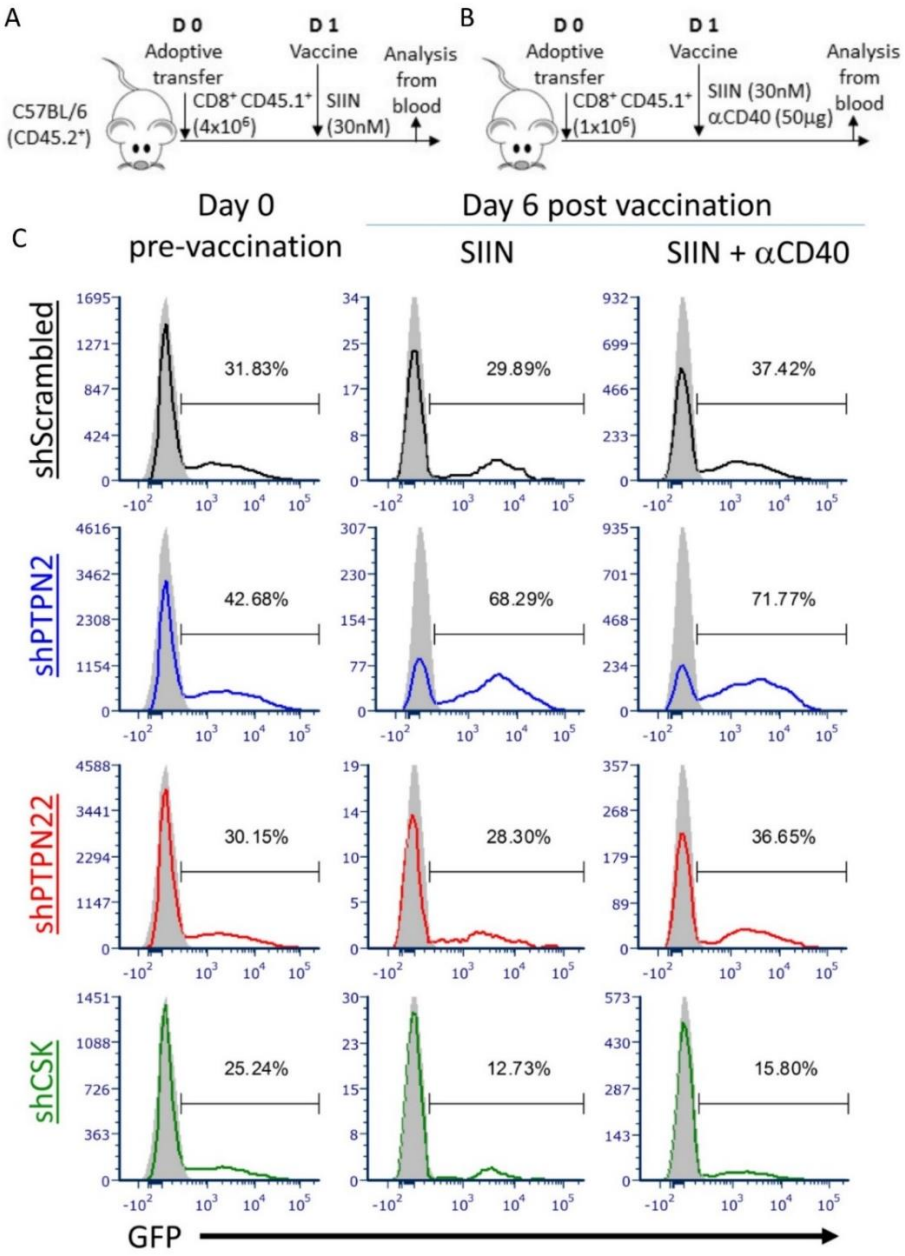

Figure 5.8: The impact of PTPN2, PTPN22, or CSK knockdown for CTL in vivo response to peptide vaccination. Naïve OT-I splenocytes were activated and transduced with shPTPN2, shPTPN22, shCSK, or shScrambled, retrovirus following the standard protocol. Following a 5-day expansion in IL-2 (10ng/ml), refreshing cytokine daily, the T cells were transferred into WT C57BL/6 mice. A, schematic detailing the adoptive transfer of $4x10^6$ total cells followed by a peptide (30nM SIIN) vaccination on the following day. B, Schematic detailing the adoptive transfer of $1x10^6$ cells followed one day later by a peptide (30nM SIIN) vaccine with costimulation (αCD40 50μg). C, Representative histograms showing the levels of GFP^+ cells within the CD45.1+ population in peripheral blood samples on the day of adoptive transfer verses day six post vaccine, measured by flow cytometry. These data are representative of two independent experiments with four mice per group.

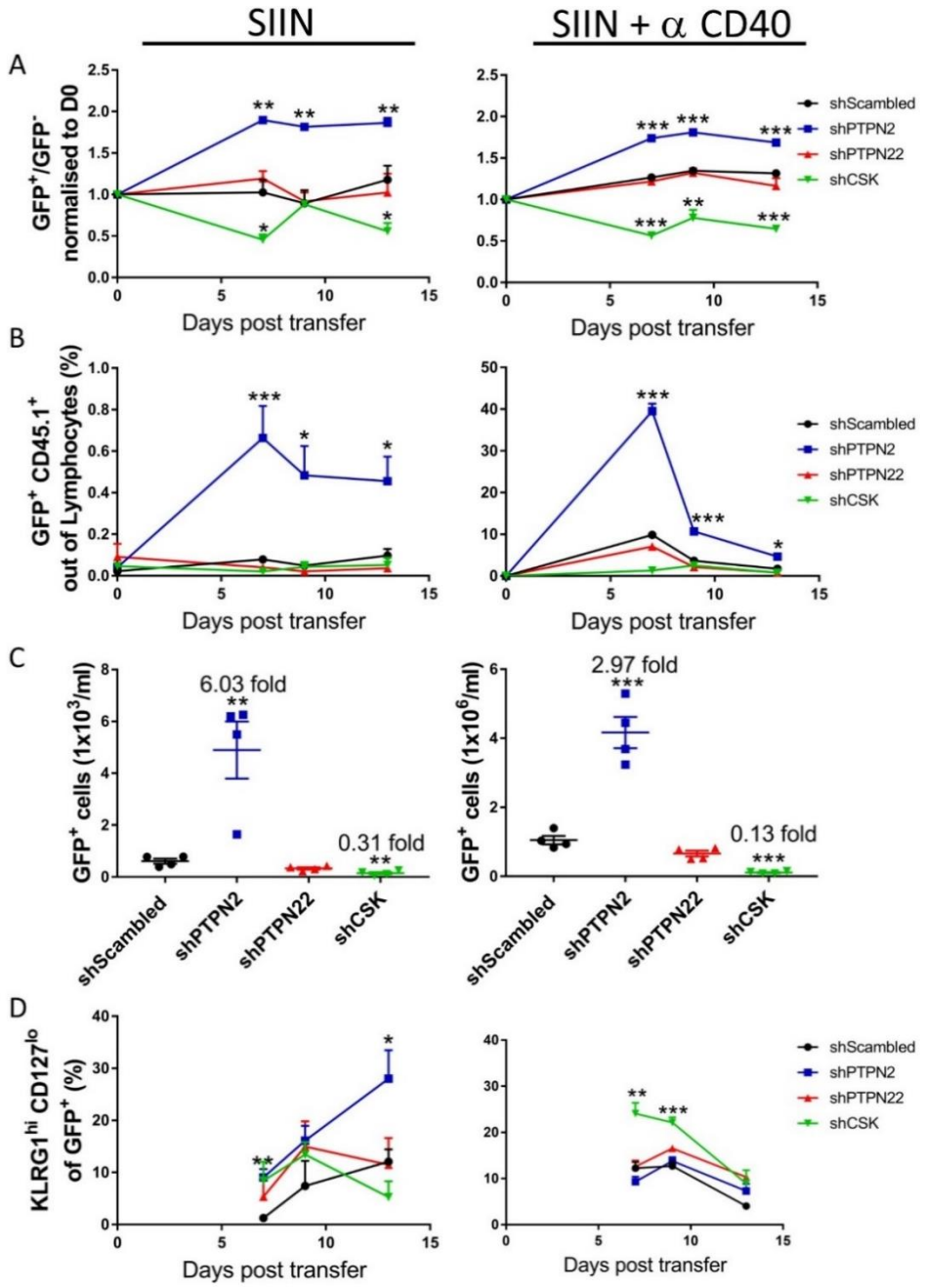

Figure 5.9: The effect of PTPN2, PTPN22, or CSK knockdown on in CD8 T cells in vivo response to vaccination. The data shown here is from the experiment established in **Figure 5.8**, where cells transduced with shPTPN2, shPTPN22, shCSK, or shScrambled, were transferred to mice which were then vaccinated with a peptide (30nM SIIN) vaccine alone or with costimulation (αCD40 50μg). All data were generated from peripheral blood samples and analysed by flow cytometry. All statistics and fold changes detailed are relative to the control shScrambled group. C, fold changes values and statistics are normalised to the initial starting ratio of GFP^+ to GFP^- cells. D, The phenotype of the transduced cells (GFP^+ $CD45.1^+$) was assessed in peripheral blood samples by flow cytometry. These data are representative of two independent experiments with four mice per group.

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Next, the improved response to vaccination granted by PTPN2 knockdown in CD8 T cells was investigated by tracking proliferation kinetics and assessing granzyme B expression. To achieve this, purified transduced OT-Is, expressing shPTPN2 or shScrambled, were generated and expanded in IL-2, stained with a Celltrace dye and transferred into mice, which were then vaccinated with peptide (30nM SIIN) (**Figure 5.10A**). Spleens were then taken on days two and three post vaccination to evaluate early response kinetics. Two days post vaccination, PTPN2 knockdown did not significantly influence the total number of GFP^+ cells or dilution of the celltrace dye (**Figure 5.10B and C**). However, by day three post vaccination, a significantly enhanced number of GFP^+ cells (8.7-fold increase) was observed for the PTPN2 knockdown group (**Figure 5.10D**). Additionally, expression levels of granzyme B, although low, were significant enhanced (2.4-fold increase) by the knockdown of PTPN2 on day three post vaccine (**Figure 5.10E and F**). By this time point celltrace dye was entirely diluted for both groups so no direct comparison of proliferation could be made. These data validated the finding that PTPN2 knockdown can be used to improve the *in vivo* accumulation and expression of an effector molecule in CD8 T cells in a vaccine setting.

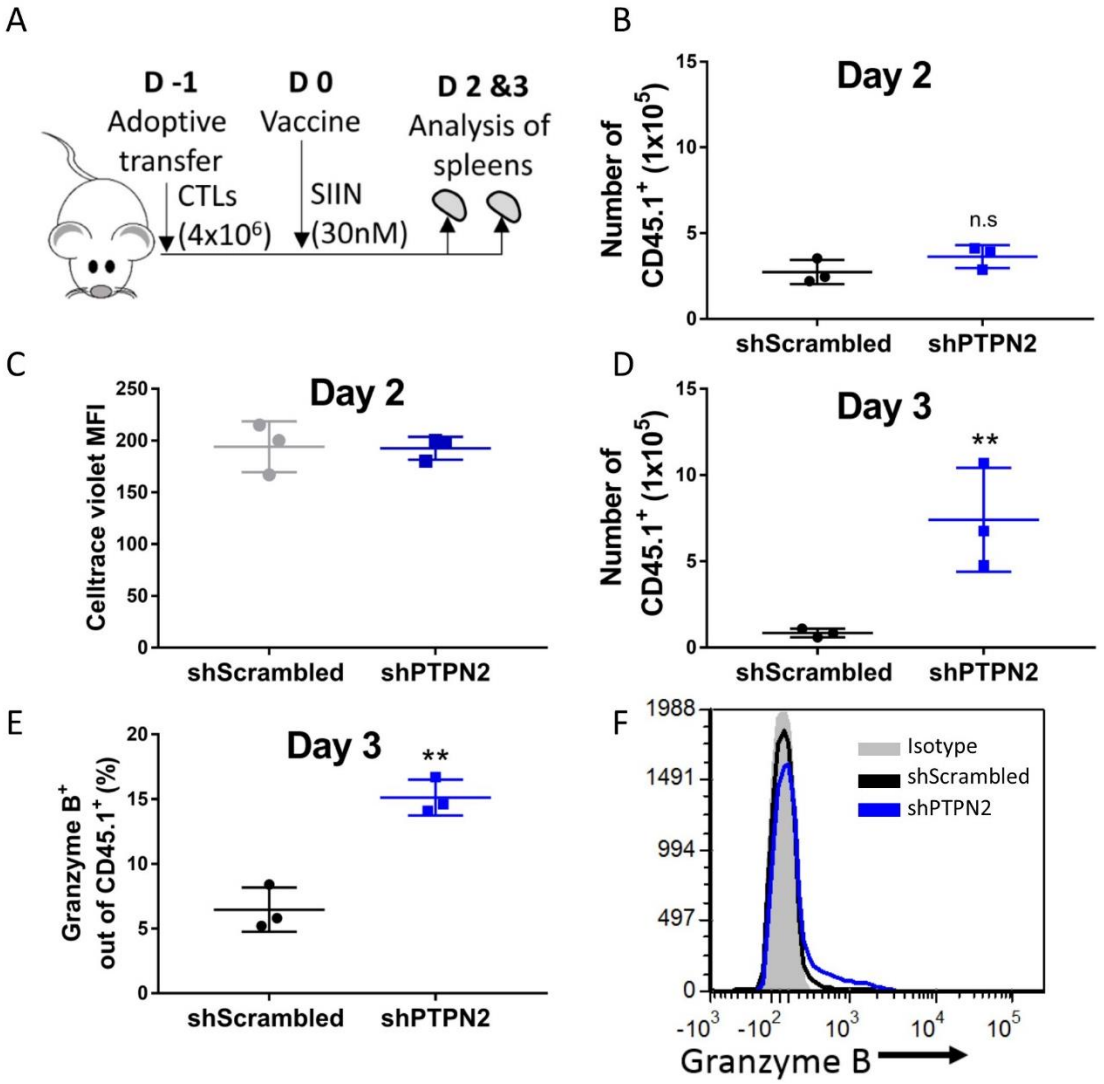

Figure 5.10: Impact of PTPN2 knockdown on in vivo CD8 T cell response to peptide vaccination. Naïve OT-I splenocytes were activated and transduced with shPTPN2 or shScrambled retrovirus following the standard protocol. The transduced CD8 T cells were purified by FACS (GFP^+ $CD4^-$) and expanded for five days in IL-2 (10ng/ml) refreshing cytokine daily. Transduced cells were stained with celltrace violet dye before transfer. A, Schematic details transfer protocol where $4x10^6$ transduced cells were transferred into WT C57BL/6 mice, which were then vaccinated on the following day with peptide (30nM SIIN.) B and C, Two days post vaccination spleens were taken to assess the number of transferred cells and dilution of cell trace dye by flow cytometry. D-F, Three days post vaccine spleens were taken to assess the number of cells and expression of granzyme B by flow cytometry. F, A representative example of flow cytometry data for granzyme B expression. This experiment was conducted once, with three mice per group of each time point.

Finally, the impact of PTPN2 and PTPN22 knockdown on OT-I T cell-mediated anti-tumour immunity was assessed in a stringent ACT model, without irradiation or vaccination. For this experiment, transduced OT-I T CD8 T cells were purified by FACS and expanded in IL-2 for five

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days, at which point $2x10^6$ CTLs were transferred into mice bearing one day established EG7 tumours. The results here demonstrate that the knockdown of PTPN2 in CD8 T cells offered improved anti-tumour immunity, significantly delaying tumour growth compared to both control groups, without ACT or treatment with control transduced cell, as well as the group receiving shPTPN22 transduced cells (**Figure 5.11A-C**). The benefit of PTPN2 knockdown was clearly seen from the average size of tumours for the groups at day 25 post tumour challenge: shScrambled - 214mm^2, shPTPN2 - 65mm^2, shPTPN22 - 183mm^2, Naïve control - 211mm^2 (**Figure 5.11B**). This delay in tumour growth translated directly into significantly improved survival duration, with the knockdown of PTPN2 conferring a median survival of 35 days, compared to 23-25 day median seen for the other groups (**Figure 5.11D**). The improved tumour control coincided with a dramatic expansion of the cells expressing the shPTPN2 between day 0 and day 6 post adoptive transfer, measured in peripheral blood samples (**Figure 5.11E**). In comparison, no expansion was observed within the shPTPN22 or shScrambled control group within this time frame. As such there was a significantly greater proportion of transferred cells out of total CD8 T cells for shPTPN2 group relative to the shScrambled control group on day 6 post transfer (**Figure 5.11E**). By day 15 post transfer, the PTPN2 knockdown cells had contracted but were still present at significantly higher levels compared to the control (**Figure 5.11D**). At this stage, over 60% of the cells within the shPTPN2 group displayed a memory phenotype ($CD127^+$ $KLRG1^-$) (**Supplementary figure 5.7**). However, with only low frequencies of transferred cells detectable in the control group it was impossible to accurately compare this trait. As the transfer of $2x10^6$ CTLs with the knockdown of PTPN22 did not significantly affect tumour control, the survival of the mice or *in vivo* expansion of transferred cells, it was not investigated further. For these experiments, no off-tumour toxicities were observed for any ACT treatment.

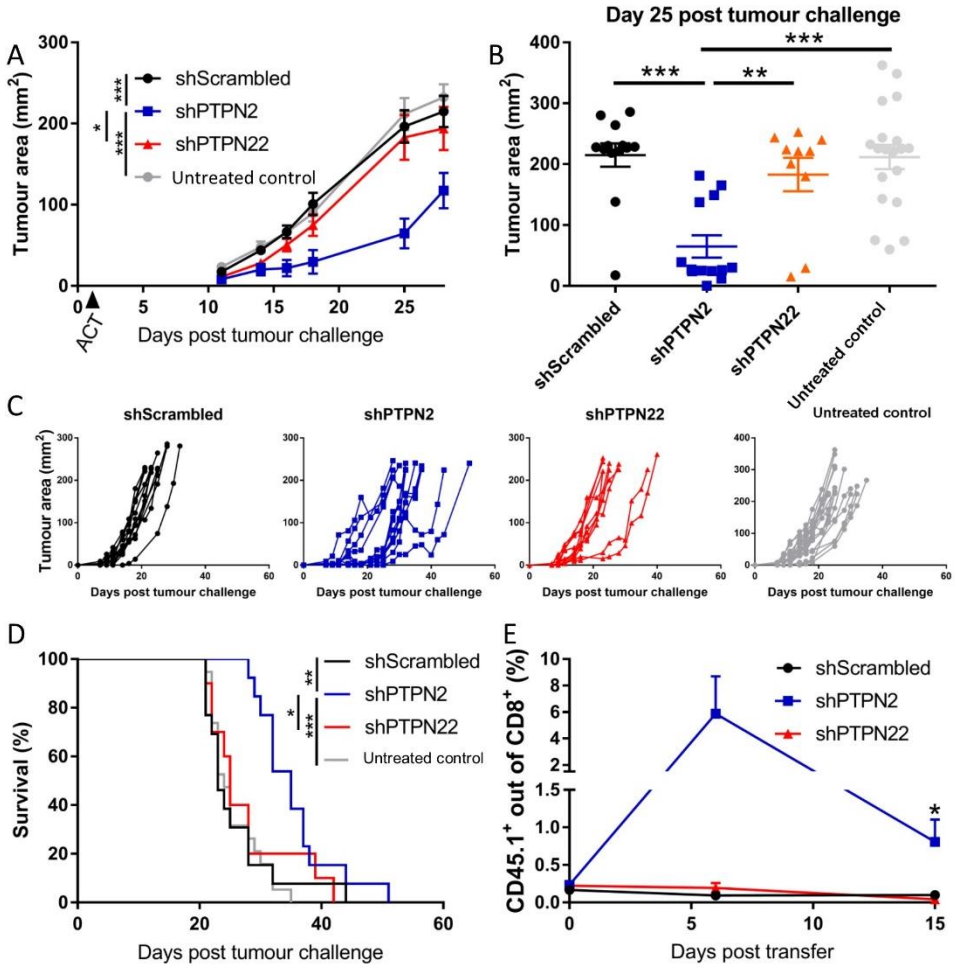

Figure 5.11: Impact of PTPN2 or PTPN22 knockdown on ACT. Naïve OT-I splenocytes were activated and transduced with shPTPN2, shPTPN22 or shScrambled retrovirus following the standard protocol. The transduced CD8 T cells were purified by FACS (GFP^+ $CD4^-$) and expanded for five days in IL-2 (10ng/ml) refreshing cytokine daily. Following the expansion, $2x10^6$ cells were transferred into WT C57BL/6 mice bearing one day established EG7 tumours. A, Average tumour size. B, tumour size at day 25 post tumour challenge. C, Tumour size of individual mice are shown over time. D, Survival duration of mice. E, Frequency of transferred cells in peripheral blood samples, measured by flow cytometry. Data are pooled from two independent experiments which each had N=5-8 mice per group.

A second experiment was set up to explore whether the benefit granted by the PTPN2 knockdown could be maintained within an even more stringent setting where a lower number of T cells were used for ACT. To assess this, purified transduced OT-Is, expressing shPTPN2 or shScrambled, were expanded in IL-2 for five days before $1x10^6$ CTLs were transferred to WT C57BL/6 mice bearing one day established EG7 tumours. Again, the knockdown of PTPN2 significantly improved the control of tumour growth compared to the naïve control group and the shScrambled transduced control group (**Figure 5.12A-C**). The kinetics of the tumour control was delayed compared to the earlier experiment using a higher transfer number, yet a benefit of PTPN2 knockdown still was

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clearly seen from the average sizes of tumours at day 25 post tumour challenge: shScrambled - 189mm^2, shPTPN2 - 98mm^2, Naïve control - 230mm^2 (**Figure 5.12B**). As such, there was a significant benefit to the survival duration of the shPTPN2 group compared to either of the control group (**Figure 5.12D**). The transferred cells remained at a very low frequency until at least day 15 post transfer, yet at day 24 post transfer there was an expansion for both the shScrambled group (approximately 10-fold) and shPTPN2 group (approximately 100-fold) was observed (**Figure 5.12E**). At this time point, the frequency of transferred CD8 T cells in peripheral blood was significantly higher for cells possessing the PTPN2 knockdown compared to the shScrambled group. Interestingly, the delay in tumour growth kinetics appeared to be correlated with the late expansion of transferred T cells observed in this experiment.

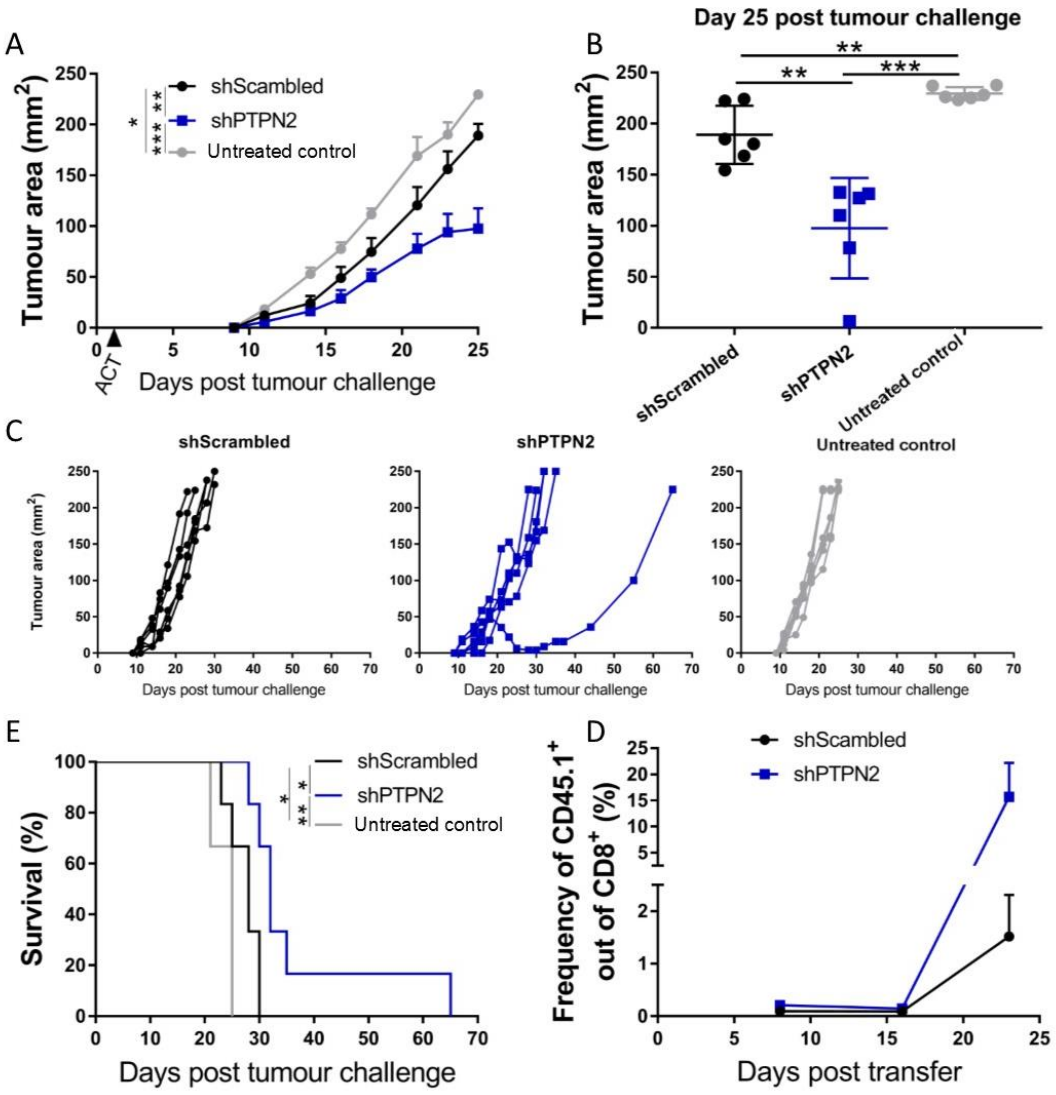

Figure 5.12: Impact of PTPN2 knockdown on ACT with low transfer number. Naïve OT-I splenocytes were activated and transduced with shPTPN2 or shScrambled retrovirus following

the standard protocol. The transduced CD8 T cells were purified by FACS (GFP^+ $CD4^-$) and expanded for five days in IL-2 (10ng/ml) refreshing cytokine daily. Following the expansion, $1x10^6$ cells were transferred into WT C57BL/6 mice bearing one day established EG7 tumours. A, Average tumour size. B, tumour size at day 25 post tumour challenge. C, Tumour size of individual mice were measured over time. D, Survival duration of mice. E, Frequency of transferred cells in peripheral blood samples, measured by flow cytometry. Data are from one experiment with N=6 mice per group.

Taken together these data show that the knockdown of PTPN2, PTPN22, and CSK have a similar effect on the responsiveness to TCR signalling *in vitro* with increased IL-2 production. However, in an *in vivo* setting, only the knockdown of PTPN2 improves responsiveness to vaccination, with the knockdown of CSK actually proving detrimental to the accumulation of CD8 T cells. In the preclinical ACT model, the knockdown of PTPN2 was shown to improve accumulation of cells and afforded better control of tumour growth. This work validates PTPN2 as a target for disruptions during ACT to achieve improved T cells responsiveness and anti-tumour immunity.

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5.3 Discussion

There is great potential for utilising genetic modifications to improve the range and efficacy of ACT by increasing the sensitivity of T cells and facilitating more effective tumour-specific responses in immunosuppressive conditions [115, 345]. With tools for introducing shRNA to induce effective knockdowns, elucidating which proteins are appropriate targets for disruption in the context of ACT is a growing area of research. Within this chapter, I demonstrate that for *ex vivo* expanded CD8 T cells the knockdown of PTPN2, PTPN22, and CSK improves IL-2 production following TCR stimulation. The PTPN2 knockdown was then shown to enhance both granzyme B expression and the accumulation of CD8 T cells following *in vivo* vaccination. Superior tumour control in a murine model could also be achieved following adoptive transfer of CD8 T cells bearing the PTPN2 knockdown, highlighting the translational potential of this genetic modification.

The first line of investigation explored how PTPN2, PTPN7, or PTPN22 knockdown impacted the function of T cells following cytokine-mediated expansion. The expansion protocols used here followed clinically relevant methods by allowing T cells to proliferate in IL-2 to achieve the large numbers required for therapy [258]. Signalling pathways activated downstream IL-2 receptor engagement could have been affected by the knockdown of PTPN2 and PTPN22, as the disruption of these factors has previously been shown to enhance STAT5 phosphorylation [118, 145]. STAT5 activation, which falls downstream of both IL-2 and IL-15 signalling pathways, is linked to differentiation and the survival of cells through control of proteins such as BCL2 [401]. However, the results here show that the knockdown of PTPN2, PTPN7, or PTPN22 did not affect phenotypic differentiation following IL-2 or IL-15 mediated expansion of CD8 T cells. It is possible that STAT5 signalling did not impact on the differentiation or expansion of T cells in this context. Alternatively, the increased STAT 5 signalling described by the previous studies with $PTPN2^{-/-}$ and $PTPN22^{-/-}$ T cells may have been an artefact of higher levels of CD25 expression following the activation of these cells [116, 145]. The latter is particularly likely for PTPN22, which has not been shown to act on factors associated with STAT5 signalling directly. As the PTPN knockdowns in this chapter were introduced following the activation of the cells, this explains why the levels of CD25 were unaffected and subsequent interactions with IL-2 were also unchanged.

Each of the phosphatases evaluated in this report have been shown to interact with factors downstream of the TCR. PTPN2 and PTPN22 directly dephosphorylate Lck and PTPN7 can

suppress ERK2 to some extent [100, 101, 129]. While PTPN2 and PTPN22 have been shown to limit TCR signalling and activation of naïve T cells, especially following weak stimulation, the consequence of the shRNA-mediated knockdown on the ability of CTLs to respond to antigen is yet to be evaluated. Here it was shown that the knockdown of PTPN2, PTPN7, and PTPN22 had no impact on the expression of IFNγ or TNFα in response to peptide stimulation across a broad range of affinities and concentrations. While published data have shown improved IFNγ or TNFα expression upon stimulation of naïve CD8 T cells deficient for PTPN22 [100], the novel setting described in this chapter illustrates that the same is not true for CTLs with a PTPN22 knockdown. Here, antigen stimulation was shown to increase IL-2 expression following the knockdowns of PTPN2 or PTPN22. While previous reports have shown that PTPN2 and PTPN22 discriminate weak from strong TCR interaction [100, 116], this phenomenon was not shown to hold true for *ex vivo* expanded CTLs, as knockdown of these phosphatases did not favour responses to weak stimulation. While higher levels of IL-2 were generated following the stimulation of cells with high-affinity peptides, the proportion of IL-2 increased by the PTPN2 knockdown was not influenced by the strength of stimulation. Interestingly, the CTLs possessing the PTPN2 or PTPN22 knockdown have increased mRNA for IL-2 as well as TNFα and to a lesser extent IFNγ. Because no difference was observed in IFNγ and TNFα at the protein level it is likely that the post-translational regulation of IFNγ and TNFα limited their expression, whereas the increase in IL-2 protein may indicate that translation is much more sensitive in relation to mRNA levels for this cytokine. It is also possible that an increase in TCR signals promoted factors that selectively increased the stability of certain transcripts. For example, phosphorylation of NF90 at Ser647 following T cell stimulation would selectively stabilise IL-2 mRNA [402]. Given that there was no change in granzyme B expression or the production of IFNγ and TNFα upon restimulation, it is unsurprising that the *in vitro* killing ability of CTLs were not affected by PTPN2 or PTPN22 knockdown.

As the inhibition of the phosphatases that limited Lck activation had been shown to improve TCR responsiveness it was relevant to expand this research to study the knockdown of CSK, a kinase that limits Lck activation through a different mechanism. The knockdown of CSK in OT-I cells gave a very similar response to the knockdown of PTPN2 and PTPN22, having little impact on the differentiation phenotype following IL-2 or IL-15 expansion, and inducing increased IL-2 production following peptide stimulation regardless of peptide affinity. Again, no changes in TNFα or IFNγ production were observed. Given these results, the knockdown of PTPN2, PTPN22, and CSK all appeared to have similar effects, increasing IL-2 expression following TCR stimulation

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therefore it is likely that the shared trait of LCK inhibition is the mechanism responsible for the observed increase in IL-2 following stimulation.

Previous studies have illustrated how the disruption of PTPN2 [116], PTPN2 [100], or CSK [156], can result in enhanced *in vivo* proliferation following TCR stimulation. However, this trait is yet to be evaluated for IL-2 expanded CTLs and using a knockdown system relevant for ACT. To test the functional consequence of these knockdowns *in vivo*, vaccination protocols were employed either using peptide stimulation with or without the addition of strong costimulation. In these settings, CSK knockdown proved detrimental to the accumulation of OT-I cells with increased KLRG1 expression suggesting terminal differentiation resulted in poor T cell survival. The knockdown of PTPN22 had no functional impact on the accumulation or differentiation of the cells. In contrast, the knockdown of PTPN2 dramatically improved accumulation of T cells. Interestingly, in both vaccination settings, the knockdown of PTPN2 gave a bystander effect, aiding the accumulation of total OT-I population including the co-transferred non-transduced population. It is possible that the improved ability of T cells possessing PTPN2 knockdown to produce IL-2 following TCR stimulation can facilitate greater autocrine and paracrine stimulation in response to the vaccine. However, this is unlikely to be the sole factor as the knockdown of PTPN22 and CSK can similarly upregulate IL-2 upon TCR stimulation. Therefore, it is likely that the PTPN2 knockdown influence other signalling pathways disparate from PTPN22. For example, PTPN2 knockdown may have led to either increased CD25 expression following stimulation or enhanced STAT5 signalling, both of which could create positive feedback loops with IL-2 stimulation during the *in vivo* response [118]. The effect of PTPN2 knockdown was most pronounced with the vaccination setting using peptide alone for stimulation. In this setting the PTPN2 knockdown afforded increased granzyme B expression illustrating an improved ability to maintain effector function. Together, these findings show the potential utility for the knockdown of PTPN2, particularly in the context of improving T cell stimulation in the tumour microenvironment where costimulatory signals may not be present at high levels.

Finally, there was a clear improvement in anti-tumour immunity achieved by the knockdown of PTPN2 in transferred CD8 T cells, which was not observed for the knockdown of PTPN22, with enhanced control of tumour growth and subsequent survival duration by the mice. Notably, therapy correlated with better accumulation of transferred cells *in vivo*. These findings are in accordance with the previously published finding where the knockdown of PTPN2 in T cells

resulted in substantial accumulation at the tumour site [113]. When the number of transferred cells used of ACT was lowered, the kinetics of the response granted by the knockdown of PTPN2 was altered, but a therapeutic benefit was still observed. As the ACT model used for these experiments did not rely on sublethal irradiation to generate a lymphopenic environment or vaccination strategy, this avoided misinterpreting results based on altered responses to these factors. However, it is likely that lymphopenic preconditioning of mice would have synergised with the transfer of CD8 T cells possessing a PTPN2 knockdown, as previous studies have shown that LIP is enhanced in cells lacking this phosphatase due to improved low-affinity interactions with self-peptide [96]. However, this hypothesis would need to be rigorously evaluated in a preclinically relevant setting, as the finding from this chapter highlight that CTLs with a PTPN2 knockdown do not show increased responsiveness to low-affinity peptides as previously described for naïve cells lacking PTPN2 [116].

It is clear from the work here that the knockdown of PTPN2 for ACT setting could synergise with vaccination strategies to improve the accumulation of T cells *in vivo*. With such optimisations, anti-tumour responses may be achievable with the use of fewer cells than in current protocols. This could derive benefits from the shorter length of time required for *in vitro* expansion of cells, resulting in lower levels of terminal differentiation and faster re-infusion of T cells to the patient. As the therapy shown in this report only achieved transient delays in tumour growth, it would be interesting to see how additional vaccination strategy could be utilised to improve the ACT therapy with the goal of achieving permanent regressions. It would also be of interest to explore whether the synergy of ACT and αPD-1 treatment [403] could be further improved through the knockdown of PTPN2, granting heightened T cell sensitivity in a suppressive environment. Analysing how PTPN2 knockdown functions in other transgenic TCRs models could be useful in exploring CD8 T cells response to known TAA. Moreover, the screening of PTPN2 knockdown first requires screening against other genetic modifications, such as the knockdown of SOCS-1 [404], Cish [346], and PPP2r2d [113], to determine which modification may be most favourable for specific therapy settings. For example, the knockdown of PTPN2 may be particularly relevant for settings where the recipients are made lymphopenic and vaccines can be applied, whereas the knockdown of SOCS-1 may be more appropriate for synergising with lower levels of IL-2 administration.

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One concern that has arisen from enhancing the sensitivity of CD8 T cells is the risk of off-tumour toxicity caused by adoptively transferred cells [405]. The use of high-affinity transgenic TCR targeting MART-1 and gp100 have resulted in off-tumour toxicity for 30% and 19% of melanoma patients respectively [297]. Additionally, in a recent clinical trial, utilising MAGE-A3 specific-TCR modified to have a supraphysiological affinity led to fatal cross-reactive responses in the two patients treated [406]. These cases highlight the potential risk associated with modulating the sensitivity of T cells and, as such, caution should be taken to evaluate potential off-tumour effects. The inclusion of a kill switch, such as an inducible caspase 9 [406], could be coincided for the clinical applications of ACT, allowing for the specific elimination of transferred cells in the eventuality of adverse effects. However, in each case the potential treatment benefit must be weighed against acceptable levels of toxicity.

From the data generated in this chapter, it appears unlikely that PTPN7 plays a role in TCR sensitivity following the stimulation of CTLs. Further work with a knockout mouse model would be required to establish if PTPN7 could play a more important role in naïve T cell activation due to its influence on the NFAT/AP1 pathway. While CSK knockdown altered responsiveness to TCR stimulation in a similar way to PTPN2 and PTPN22 knockdowns *in vitro*, the *in vivo* response of T cells was markedly different, suggesting that the inhibition of the phosphates improved survival pathways or that CSK interacts with factors that normally reduce terminal differentiation. Further work would be required to evaluate CSK targets to understand the mechanism responsible for this phenomenon. Future research possibilities could also include investigating why PTPN2 and PTPN22 knockdown presented differently during *in vivo* settings. Several possible factors could explain why PTPN2 knockdown in CD8 T cells provided a benefit in vaccination and ACT while PTPN22 knockdown did not. The most obvious is that PTPN2 has substrates that are not shared with PTPN22, such as JAK or STAT pathways [117, 118], which would increase the sensitivity of cells to cytokine stimulation and offer positive feedback within the immune response. Another possibility could be that PTPN2 is simply more important in controlling Lck dephosphorylation compared to PTPN22 in the setting tested here and therefore its disruption influenced the accumulation of cells to a greater extent. In either case, here it would appear that PTPN22 activity is redundant within the context of OT-I CTLs responding *in vivo* to vaccination or tumours. Most importantly, the knockdown of PTPN2 shows potential as a universal augmentation to improve CD8 T cell-function for ACT and warrants further preclinical investigation within other tumour models and synergy treatment strategies to evaluate its full potential.

Chapter 6: Final discussion

Recent developments in immunotherapy have allowed for the treatment of various malignancies with *ex vivo* expanded lymphocytes. As tools have been developed for effective genetic modifications, new options have emerged for augmenting T cells for ACT therapy. Successful applications of this technology have been achieved by redirecting T cell specificity through the incorporation of transgenic TCRs or CARs targeting common tumour-specific antigens. The platform of genetically modifying cells prior to adoptive transfers also offers a plethora of opportunities for optimising CD8 T cell survival and function, which is often compromised by prolonged *ex vivo* expansion favouring terminal differentiation or hindered *in vivo* by the immunosuppressive tumour micro-environment. Elucidating which genetic alterations can facilitate robust anti-tumour responses, whilst avoiding dramatic off-tumour toxicities, is a key goal for improving efficacy and expanding the range of cancers treatable by ACT. The data presented here illustrates that modifications can have complex and sometimes unexpected effects on the phenotype and function of T cells. As such, the overexpression of certain transcription factors associated with memory development were shown to be ineffective at promoting CD8 T cell function within an ACT setting. However, enhancing CD8 T cells response to stimulation, via the knockdown of PTPN2, improved anti-tumour immunity and highlighted the translation potential for disrupting this phosphatase during ACT therapy.

There is currently a lot of interest surrounding the use of genetic modifications for improving ACT therapy. For instance, ground-breaking work has been performed by Carl June and colleagues who have been pursuing the development of an off-the-shelf CAR T cell for cancer treatment. In a recent study, lentiviral transduction followed by multiple rounds of electroporation generated CAR T cells had the expression of HLA class I, TCR, and PD1 disrupted [344]. Although there is still much work to be done in this area, this marks the first steps towards reducing alloreactivity and avoiding graft-versus-host disease for a universally applicable CAR T cell therapy. Furthermore, this work showed that the disruption of PD1 enhanced the *in vivo* anti-tumour ability of CAR T cells, demonstrating the clear benefit of removing inhibitory signals that often limit T cell function in the tumour microenvironment. The development of CARs that optimise the performance of the transduced T cells has also been progressing incrementally over recent years with the introduction of intracellular costimulatory domains. With second-generation CAR T cells it was found that the use of a 4-1BB signalling domain improved survival and promoted memory phenotypes compared to a CD28 domain. Many studies are now focused on improving T cells

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persistence *in vivo* as the development of a memory phenotypes have been associated with sustained anti-tumour immunity. One recent example of this is the use of an ICOS intracellular domain on a second-generation CAR improving the *in vivo* persistence of CD4 T cells, which in turn enhances the persistence of CD8 cells and boosted anti-tumour immunity in a murine model [407]. With the possibility of multiplex genome editing alongside CARs or transgenic TCRs, there is a need to uncover genetic modifications that will translate to T cells having improved survival and the ability to overcome immunosuppressive factors during ACT therapy.

For my PhD, I aimed to elucidate how certain genetic modifications would impact the function of CD8 T cells and to assess if they could benefit ACT therapy. Current methods for targeting TAA often rely on common self-antigens, which are difficult to generate effective anti-tumour immunity against without incurring off-tumour toxicity. For example, targeting MART-1 or MAGE-A3 can be effective for the treatment of melanoma, but often result in off-tumour toxicity in many other tissues [297]. Even with recent success stories, many cancer patients do not respond to ACT therapy, as tumours continuously adapt to evade detection or destruction. Attempting to enhance the efficacy of ACT by augmenting transgenic TCRs to have a super-physiological affinity towards self-antigens can be extremely dangerous with potentially lethal off-tumour toxicity [303]. This drawback may limit the range of treatable cancers and raises important safety concerns. With improving technologies a solution may be to sequence individual cancers to find multiple neoantigen targets for each patient [310]. While T cell clones can be generated against unique neoantigen, the process of augmenting each individual TCR to achieve high-affinity interactions would be costly and time-consuming. A more efficient approach would be the use of universal generic modifications to improve the function of any neoantigen-specific CD8 T cells. As such, finding factors to improve T cells survival or sensitivity may be the key to optimising anti-tumour immunity.

Within my PhD, I investigated two approaches for augmenting T cell function prior to ACT. Firstly, improving the formation of T cells with a memory phenotype via the forced expression of certain transcription factors. The second tactic was enhancing the sensitivity of CD8 T cell stimulation through the knockdown of TCR inhibitory phosphatases and a kinase. To determine the translatable potential of these modifications, the differentiation and function of transduced T cells were assessed *in vitro*. This was followed by selectively testing of promising factors within *in vivo* models of vaccination and ACT therapy against tumour bearing mice.

Here, I first developed new tools and transduction methodologies to genetically modify CD8 T cells in a pre-clinically relevant system. The benefit of using IL-12 during CD8 T cell activation to substantially increase transduction efficacy was clearly demonstrated. This work was later corroborated by an independent group also reaching the conclusion that IL-12 offered the greatest benefit to retroviral transduction compared to many other cytokines [363]. Following this, several ACT models were established for the treatment of EG7 tumours with transduced OT-I T cells that had been expanded in IL-2 to achieve large numbers. These models evaluated the impact of vaccination with or without the use of sublethal irradiation preconditioning. A high number of T cells were required to mediate anti-tumour immunity but responses from lower T cell transfers could be achieved with the use of sublethal irradiation, vaccination, or less established tumours. These protocols offered appropriate platforms to test whether genetic modifications offered therapeutic benefit to ACT therapy.

My first research aim was to endow CD8 T cells with characteristics of a memory phenotype to facilitate improved survival and anti-tumour responses. Preclinical models have illustrated that the transfer of T cells with memory phenotypes can benefit anti-tumour immunity. For instance, a recent study has shown that the overexpression of CXCR4 by CD8 T cells enhances migration towards vascular-associated CXCL12+ cells in the bone marrow [408]. This allows for greater levels of IL-15 mediated homeostatic proliferation and promoted a memory phenotype with higher levels of CD62L expression following antigen priming. This was also associated with reduced PD1 expression and improved poly-functional cytokine production. These memory traits were hugely beneficial when it came to ACT treatment of lymphoma-bearing mice, as CD8 T cells over-expressing CXCR4 had greater capacity to expand and mediate tumour protection [408]. The improved persistence and functionality achieved by memory T cells makes the promotion of these cells a high priority for ACT therapy. This notion is further supported by retrospective analysis of clinical data, indicating that ACT therapy is less effective when using terminally differentiated T cells when compared to cells with memory phenotypes that have improved persistence and proliferative capabilities *in vivo.* In practice however, current clinical protocols achieving the vast number of cells required for therapy often relies on multiple rounds of stimulation and expansion of T cells in IL-2, resulting in terminal differentiation. Recently it has been demonstrated by Restifo's group that the use of an Akt inhibitor during the *ex vivo* expansion of cells can be highly beneficial to the retention of cells with a memory profile, which then provided more robust anti-tumour immunity in a murine model [290]. Promisingly, Akt inhibition does not limit the levels of *in vitro* expansion and can be combined with retroviral or lentiviral transduction [409]. The

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inhibition of Akt has been linked to sustained intra-nuclear localisation FOXO proteins, which regulate aspects of T cell homeostasis and differentiation. As such the overexpression of FOXO1 in T cells has been shown to phenocopy T cells treated with an Akt inhibitor, showing enhanced central memory formation as measured by increased levels CD62L [409]. It was therefore of interest to introduce an Akt-insensitive FOXO1 gene into CD8 T cells to test if this targeted manipulation of a transcription factor would promote functional memory development. While the transduction of CD8 T cells with CA FOXO1 conferred the phenotypic appearance of memory, with high levels of CD62L maintained in IL-2 mediated expansion, this alteration did not influence CD8 T cells response to vaccine rechallenge *in vivo* and was actually detrimental to anti-tumour immunity. It is possible that the limited migration was responsible for reduced frequency of cells being activated which caused the reduced levels of granzyme B observed. Future work could aim to investigate the precise mechanism by which FOXO1 limits anti-tumour immunity. By harvesting TILs following ACT, relative accumulation and phenotype of transduced T cells would give great insight into the role of FOXO1 on migration and effector function in the tumour setting. If these experiments yield results that indicate the inherent cytotoxicity of T cells is not affected by CA FOXO1, the investigation could continue with a more appropriate setting that does not rely on peripheral migration for therapy such as a leukaemia tumour model. If no positive data is attained from these experiments an alternative investigation could explore whether the use of shRNA mediated knockdown of FOXO1 could improve peripheral migration and effector function of CD8 T cells post-ACT. Additionally, while all the work here has focused on CD8 T cells, similar lines of research could be conducted with CD4 T cells, which can play a crucial role for anti-tumour immunity for ACT [410] and are likely to respond differently to the over-representation of certain transcription factors.

Additional factors in the Akt signalling pathway known to affect T cell differentiation into memory includes the transcription factors T-bet and Eomes. As previous studies have shown that the knockout of Eomes results in defective features for memory and cytotoxic function [58], the work here evaluated if the converse could be achieved through the overexpression of Eomes. Despite mitigating the effects of IL-2 mediated effector differentiation *in vitro*, the *in vivo* engraftment and secondary response to vaccination by transduced T cells was not improved by the overexpression of Eomes. Inherently this is not consistent with a published study, in which the overexpression of Eomes improved *in vitro* and *in vivo* accumulation response to vaccination [59]. Potentially, these differences in results could be explained by differing levels of Eomes expression achieved following T cell transduction. Theoretically, if the Eomes expression was stronger in the

system used here a dysfunctional state may have been promoted [391], while lower levels of Eomes expression may be more appropriate for promoting functional memory development. In line with the concept of the system here promoting a dysfunctional profile, the overexpression of Eomes consistently reduced the ability of cells to express IL-2 upon re-stimulation. Taken together, these data illustrate how the conventional classification of CD8 T cell subsets can be manipulated by over-representing transaction factors, but phenotypes do not necessarily correlate with the expected function for these populations.

While physiological levels Eomes and FOXO1 are important for memory development, their enforced overexpression fails to induce functional memory differentiation in a way that is beneficial to ACT. It is possible that graded or transient overexpression of these factors may be of benefit, especially during harsh *ex vivo* expansion phases to limit levels of terminal differentiation similarly to Akti treatment. However, transcriptional plasticity may be key for T cells to regain effector functionality and patrol peripheral tissues facilitate effective anti-tumour immunity. Interestingly, a similar conclusion was recently expressed by a paper published by Restifo's group who suggest a conditionally active version FOXO1 would be necessary for the context of ACT to permit trafficking to peripheral tissue [409]. To achieve this, future investigations could transduce the donor T cells with a vector containing a doxycycline-inducible promoter. This would allow for graded and temporal control of gene expression, facilitating rigorous assessment of the role of transcription factors at different stages of T cells differentiation. Future work in this area could also benefit from utilising transcriptomic analysis of transduced cells at different stages of differentiation. With improvements in technology, drop-seq analysis of individual cells within an anti-tumour response could generate maps of T cells differentiation. This would allow for a detailed assessment of over-representing transcription factors while linking these profiles to the functional outcomes.

My next set of experiments focused on inhibiting factors that limit CD8 T cell stimulation with the aim of improving anti-tumour immunity following ACT. Several preclinical studies have shown enhanced efficacy from adoptively transferring T cells that have an inhibitory pathway or receptor disrupted, including Cish [346], TGFβ type II receptor [342], FAS [342], Cbl-b [343], and PPP2r2d [345]. Here, the knockdown of several PTPNs and a PTK, linked to the inhibition of TCR signalling, were evaluated. Improved TCR T cell sensitivity through the disruption PTPN2, PTPN22, and CSK have been reported for only weak stimulation or low-affinity interactions, which is highly relevant

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for targeting low-affinity TAAs [100, 101]. However, this same facet was not observed here evaluation CD8 T cells transduced with shRNA and expanded in IL-2 to form a CTL profile. While the production of IL-2 in response to TCR stimulation was enhanced by the knockdown of PTPN2, PTPN22, and CSK, the affinity of antigens did not seem to influence magnitude of this response. Therefore, it is possible that LCK regulation is more important for governing naive T cells responses towards weak antigens compared to restimulation of CTLs evaluated here. Leading on from these finding, the impact of these various knockdowns were shown to be strikingly dissimilar in an *in vivo* vaccination setting. The knockdown of CSK limited the accumulation of transferred cells and knockdown of PTPN22 did not influence the *in vivo* response. PTPN2 knockdown alone was shown to have a positive effect on the accumulation of transduced cells, which also expressed higher levels of granzyme B and provided a bystander effect by enhancing the accumulation of non-transduced co-transferred cells. As the knockdown of PTPN2, PTPN22, or CSK was shown to have a similar response *in vitro* to TCR stimulation, it is unlikely that the enhanced production of IL-2 alone was responsible for this phenomenon. It seems likely that removal of PTPN2 mediated inhibition on another pathway, such as STAT5, provided positive signals to improve the accumulation of cells. For the future investigation of T cells possessing the PTPN2 knockdown, it would be of interest to determine the mechanism for improved responsiveness to vaccination and anti-tumour response compared to the knockdown of PTPN22. The first line of investigation should explore JAK1 and JAK3 signalling pathways following the stimulation of transduced cells with IFNγ or cytokines that bind the common gamma chain (e.g. IL-2, IL-7, IL-15 and IL-21). For example, stimulating cells with IL-2 and measuring the phosphorylation of STAT proteins over a short time course may elucidate whether the knockdown of PTPN2 offers an advantage to effector T cells immune response to stimulation downstream of factors other than the TCR.

The work here has shown that PTPN2 knockdown enhances the *in vivo* accumulation of transferred cells and improves tumour control within an ACT therapy model. These data highlight that even within a stringent setting without the use of lymphopenic preconditioning or vaccination, ACT therapy against tumours can be optimised by inhibiting PTPN2 and thereby lifting suppression on T cell stimulation. Moving on from this work, it would be important to test how impactful the PTPN2 knockdown in CD8 T cells performs in different ACT settings. Lymphodepleting mice prior to ACT would create a more translatable treatment setting and is also likely to selectivity benefit the engraftment of cells possessing the PTPN2 knockdown [96]. Furthermore, testing vaccination strategies to synergise with T cells possessing the PTPN2

knockdown will likely lead to improved levels of anti-tumour immunity. It is interesting to note that the ACT therapy here was unable induce complete remission of the EG7 tumours, even with the addition of vaccination and αCD40 stimulation resulting in huge expansion of tumour specific T cells. It is likely that the selection bias favoured the loss of $H2K^b$ SIIN expression and resulted in tumour immune evasion. This could be clarified by isolating the potentially immune escaped tumours and testing their ability to stimulate OT-I T cells compared to freshly cultured EG7 cells.

Before translating PTPN2 knockdowns into a clinical setting, it will be valuable to investigate the robustness of these results in other models and explore how this modification will perform once combined with existing treatment strategies. Many murine tumour models have been extensively profiled, such as B16, MC38, 4T-1, or RENCA. These models could be utilised to characterise settings in which the benefit of PTPN2 knockdown is maintained. Bioinformatic data could also address the question of whether the reduced PTPN2 activity is beneficial in human tumour settings by screening cancer patients with loss of function SNPs for PTPN2 and assessing levels of T cell infiltration and disease progression. An additional line of investigation would be to test the effectiveness of this knockdown in T cells transduced with existing transgenic TCRs or CARs. It will be important to understand the impact of PTPN2 regulating on Lck and cytokine signalling within these systems as these modifications are likely to remain the cornerstone of ACT modification. With the progress of multi-plex genome editing it will be possible to consider targeting multiple pathways simultaneously to provide synergistic benefits for transgenic T cells [344]. Finally, evaluating PTPN2 knockdown in ACT models alongside checkpoint blockade such as αPD-1 or αCTLA-4 may offer insight into the utility augmenting internal and external mechanisms of T cell suppression during ACT.

To conclude, disrupting proteins that limit T cells function is a valid strategy for improving the effectiveness of ACT in preclinical models, with work here highlighting the translatable potential for PTPN2 disruption in CD8 T cells. Future work will be able to further elucidate mechanisms of action and potential synergistic treatment options using this genetic modification to improve ACT therapy against cancer.

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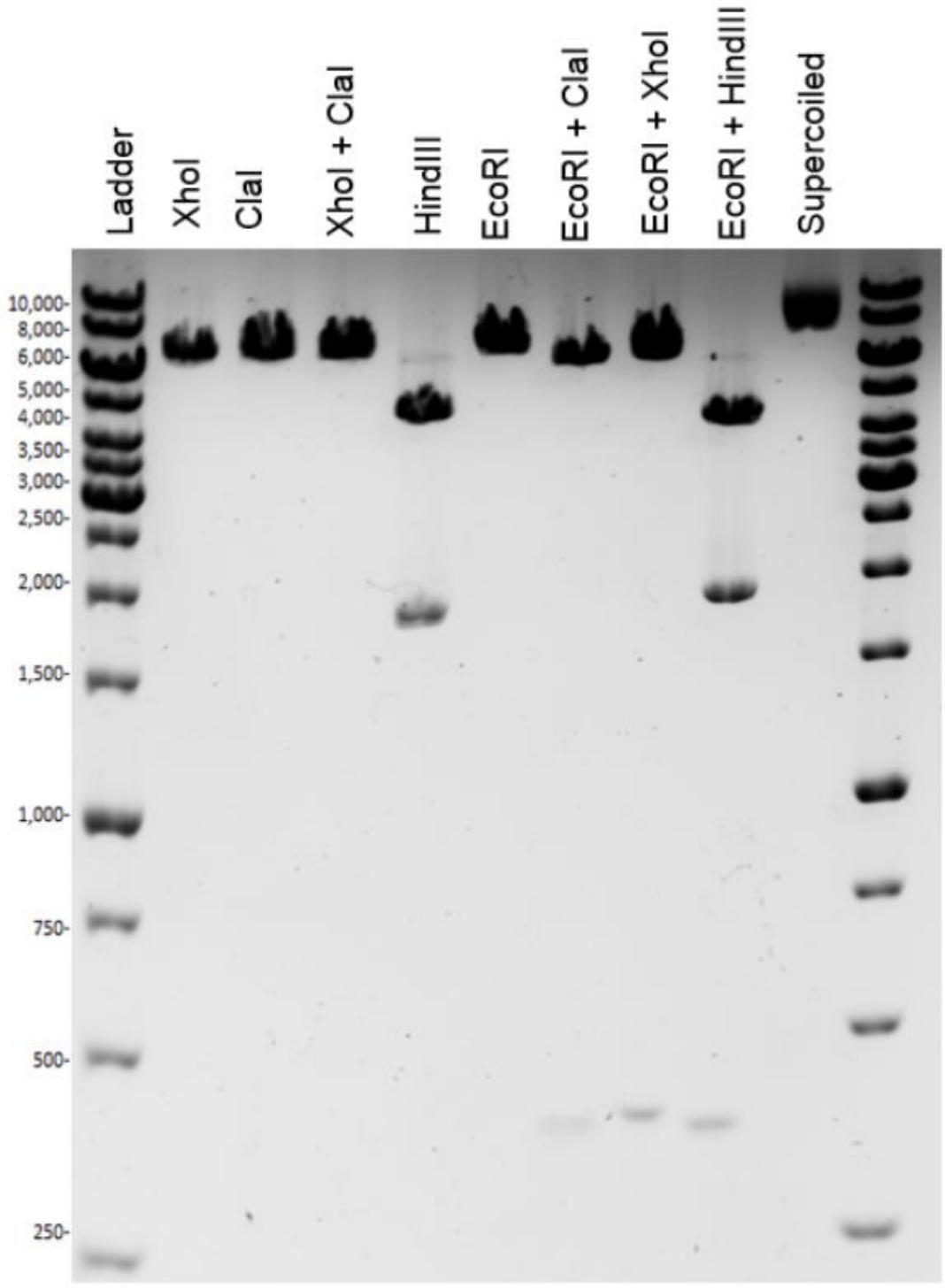
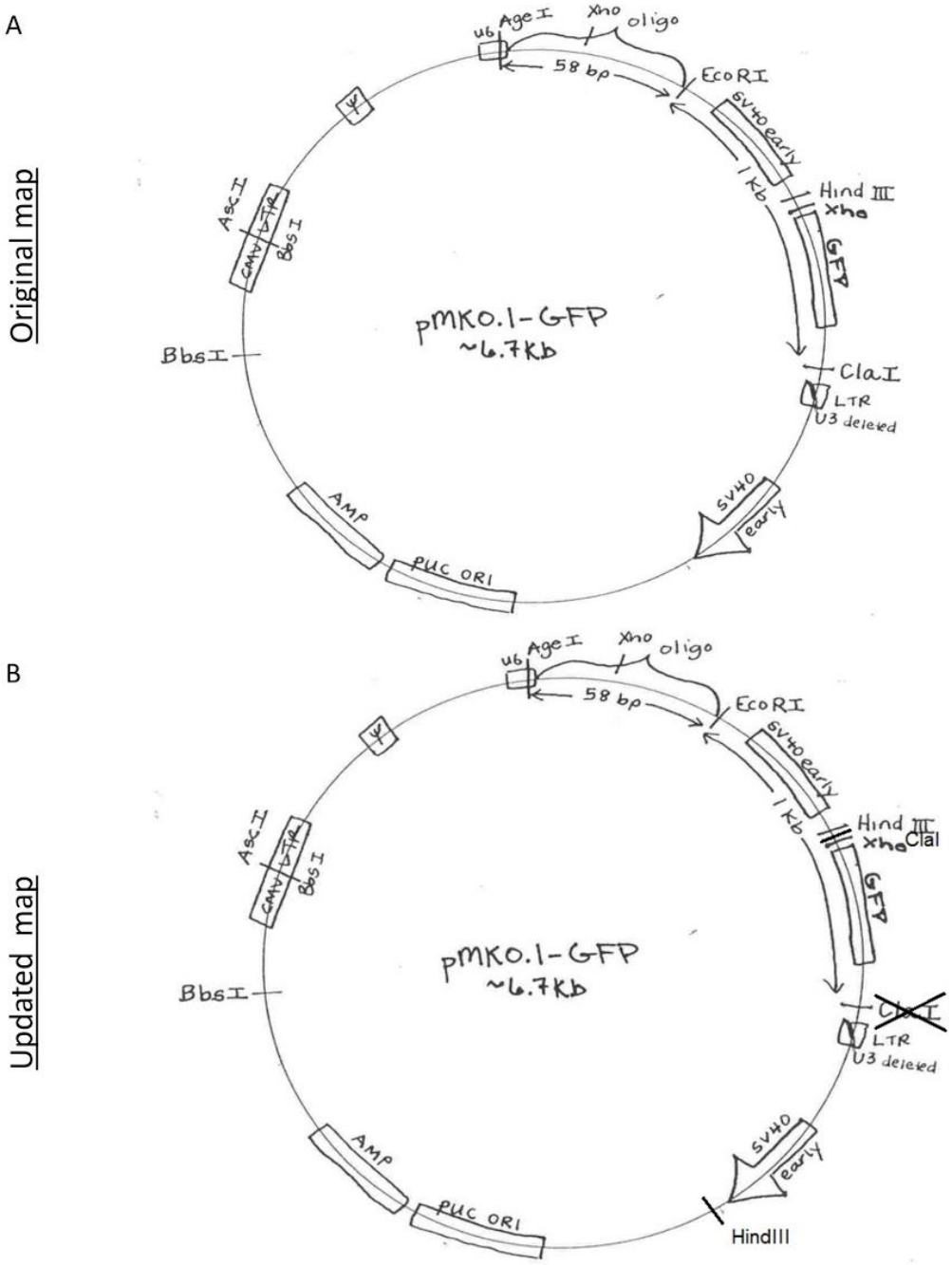
Supplementary figures



Supplementary figure 3.1: Diagnostic restriction digestion of the pMKO.1 GFP vector. Various restriction enzymes were used to determine the location and number of restriction sites present for the pMKO.1 GFP vector.



Supplementary figure 3.2: Schematic maps of the original pMKO.1 GFP map and updated pMKO.1 GFP map. A, The original map provided by Addgene. B, The new map determined by restriction digestion

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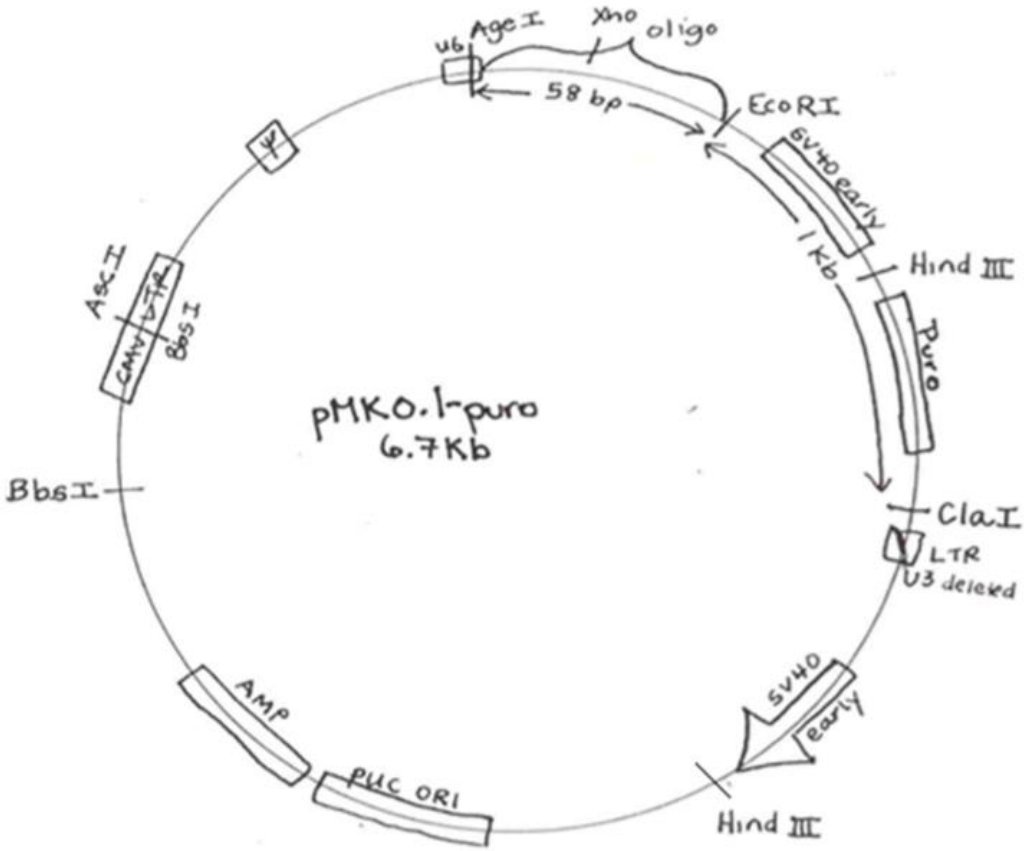
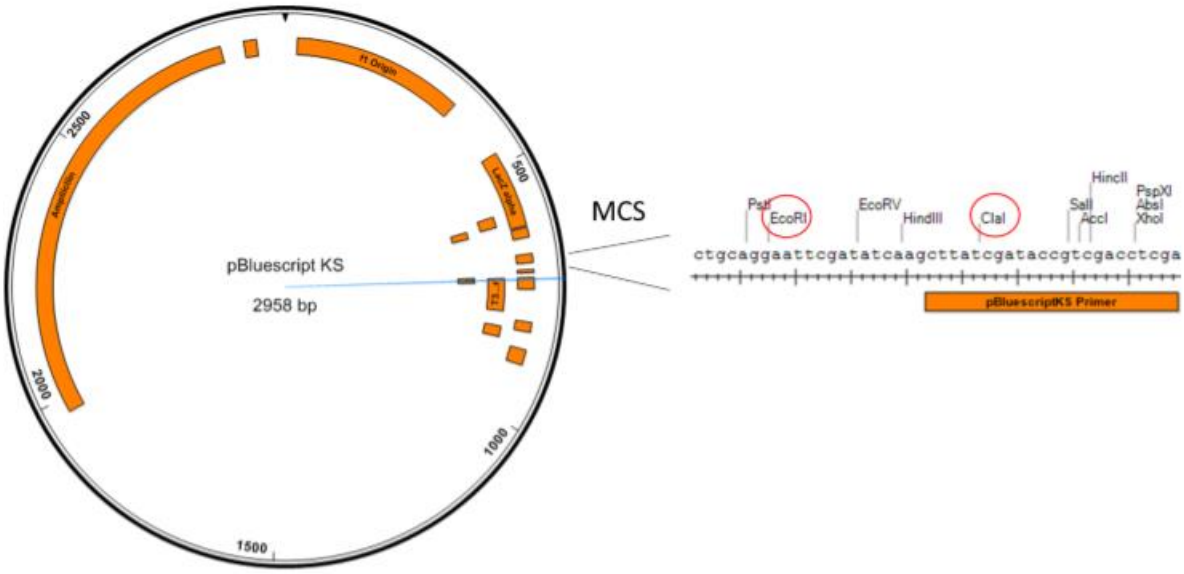
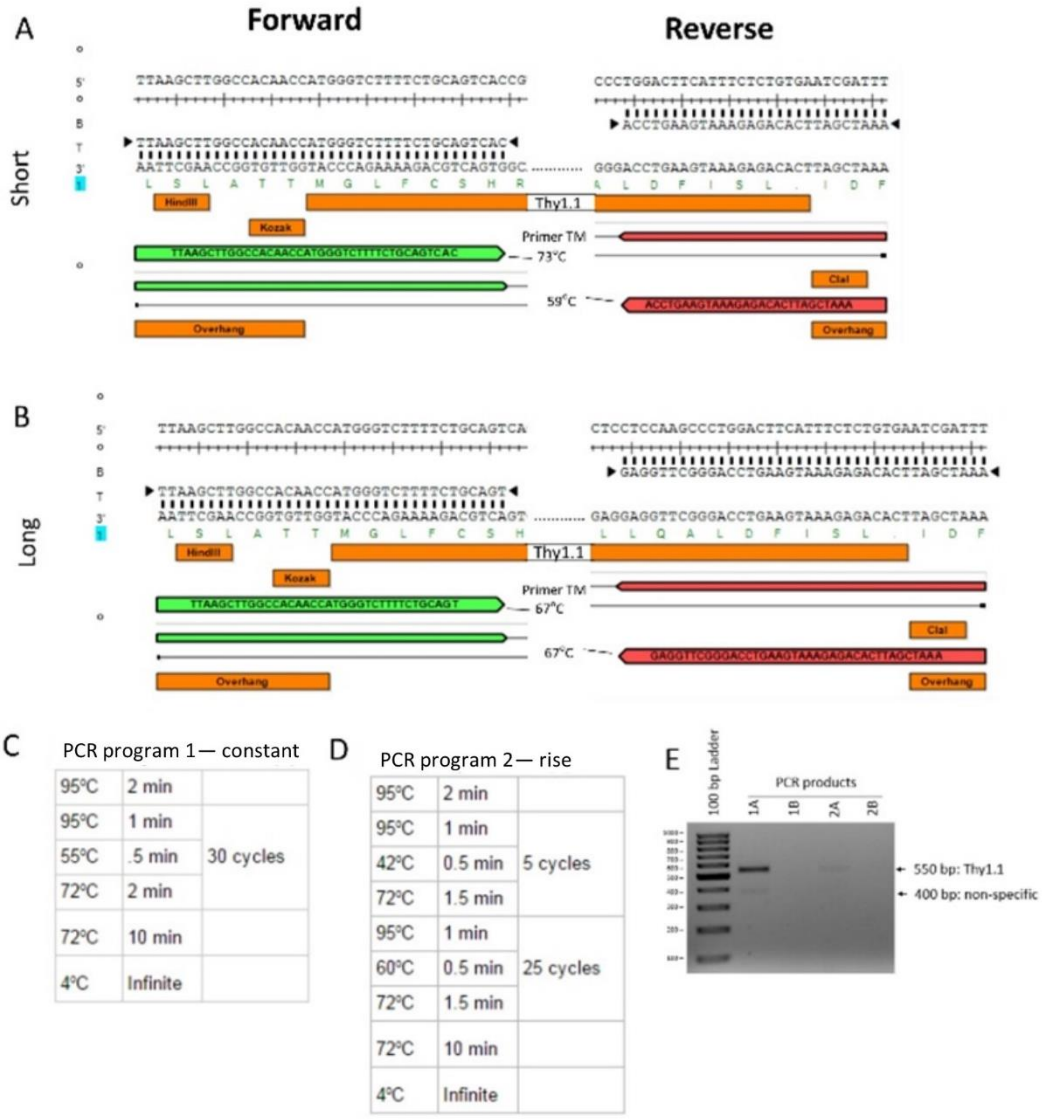


Supplementary figure 3.3: Map of pMKO.1 Puro. Provided by Addgene



Supplementary figure 3.4: Map of pBluescript KS with multiple cloning site excerpt.



C

PCR program 1— constant

95°C	2 min	
95°C	1 min	30 cycles
55°C	.5 min	
72°C	2 min	
72°C	10 min	
4°C	Infinite	

D

PCR program 2— rise

95°C	2 min	
95°C	1 min	5 cycles
42°C	0.5 min	
72°C	1.5 min	
95°C	1 min	25 cycles
60°C	0.5 min	
72°C	1.5 min	
72°C	10 min	
4°C	Infinite	

Supplementary figure 3.5: Primers and PCR programs for Thy1.1 amplification from pMiT. Two primer sets were designed for the amplification of the Thy1.1 gene from pMiT. A, primers that extend 20 bp into the Thy1.1 gene, which therefore creates a shorter reverse primer. B) In the second primer set, the reverse primer extension into Thy1.1 is longer while the forward primer was shortened to create equal TM values for both primers. C, The PCR program 1 had a constant annealing temperature of 55°C. D, The PCR program 2 rose from an annealing temperature of 42°C to 60°C after the first five cycles. E, PCR products: A1: Short primer with constant annealing temperature, 1B: Long primer with constant annealing temperature, 2A: Short primer with rising annealing temperature, 2B: Long primer with rising annealing temperature.

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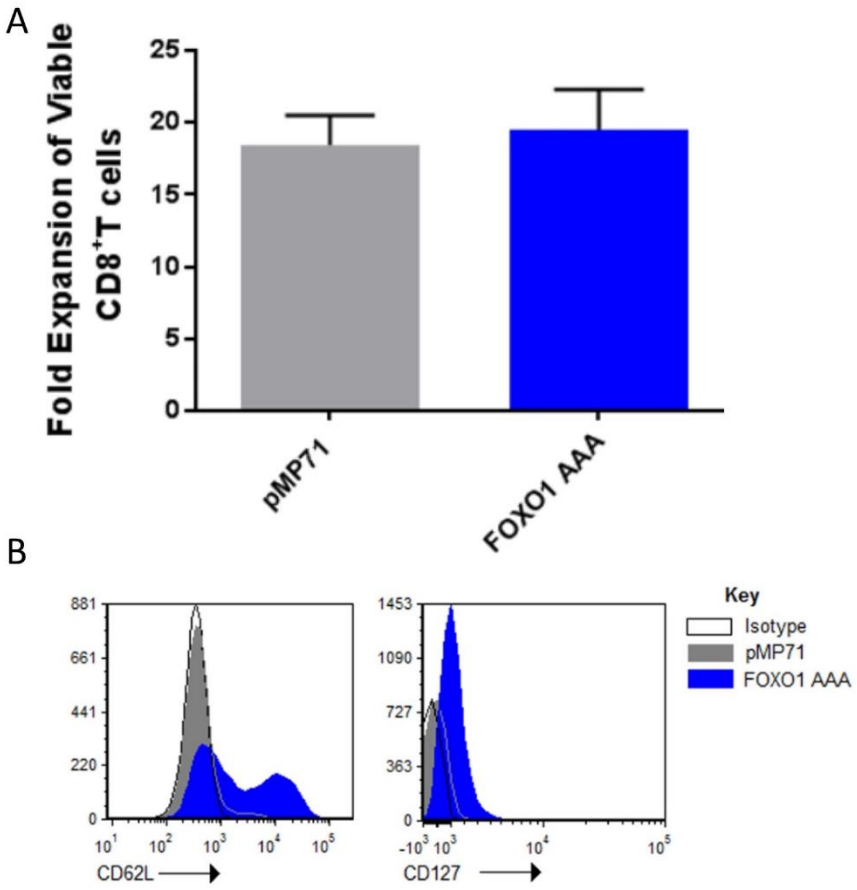
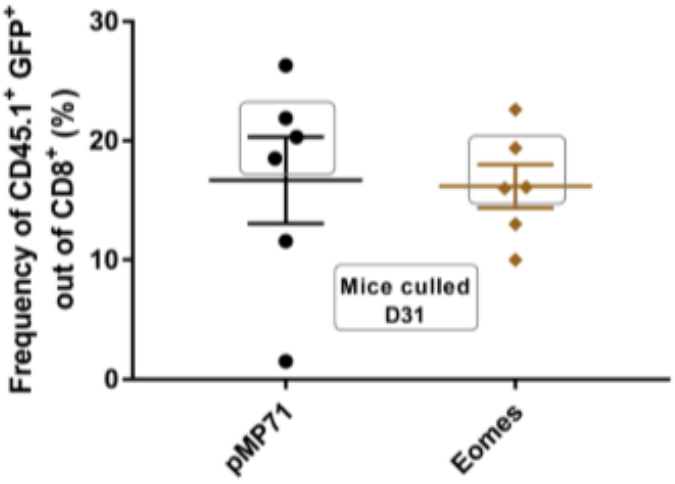


Supplementary figure 4.1: The effect of constitutively active FOXO1 on CD8 T cells expansion and differentiation. Naive OT-I (CD45.1^{+}) splenocytes were stimulated with Con-A (5μg/ml) and IL-7 (1ng/ml) for 24 hours prior to transduction with either pMP71 or FOXO1 AAA retrovirus. 48 hours post-transduction, CD4^{-} GFP^{+} cells were FACS-sorted, expanded for five days in IL-2 (10ng/ml). A, The relative expansion of transduced OT-I T cells was assessed following the five day culture in IL-2. B, At this point the expression CD62L and CD127 were assessed by flow cytometry. Data are pooled or representative from at least two independent experiments.



Supplementary figure 4.2: Mice culled at the peak of the vaccine response. Following the protocol described in **Figure 4.11**, three mice were culled on D31 post transfer, corresponding to 6 days after SIIN (30nM) /αCD40(50μg) vaccination). The frequency of CD45.1$^+$ cells was assessed by flow cytometry to determine three average mice to cull. Spleen, liver and lymph nodes were taken for analysis. Data from these are shown in **Figure 4.12** and **Figure 4.13**. This experiment was performed once.

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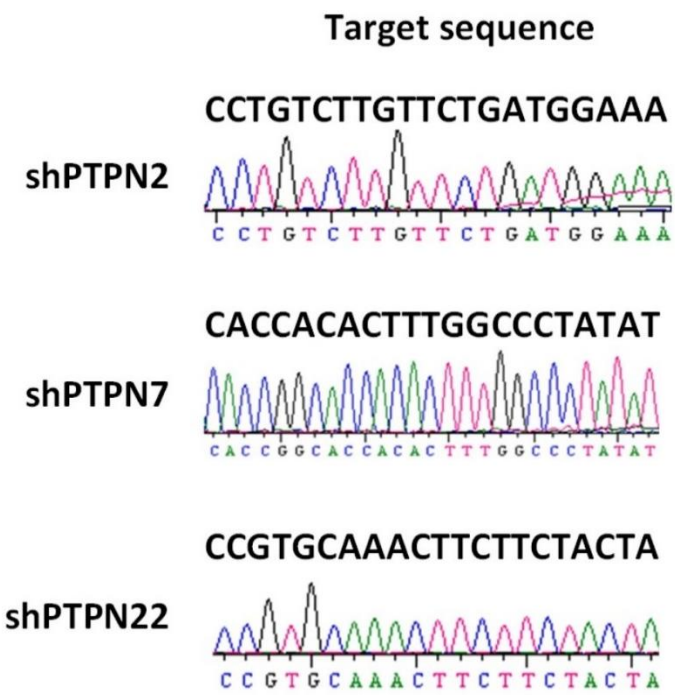
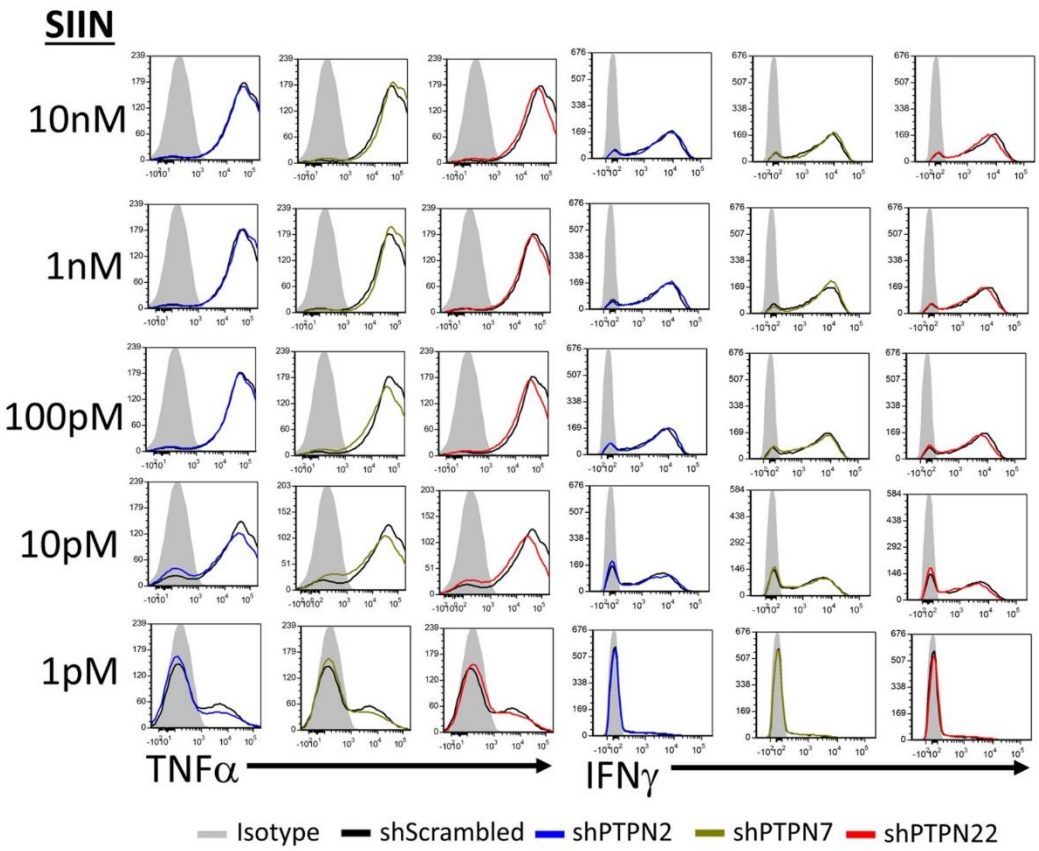


Supplementary figure 5.1: Sequenced pMKO.1 GFP vectors containing knockdown sequences. The shPTPN2, shPTPN7, and shPTPN22 plasmids were sequenced using the pLSNX5' primer.



Supplementary figure 5.2: Effect of Phosphatase knockdown on TNF and IFN production by CTLs. Representative data from **Figure 5.5 A and B.**

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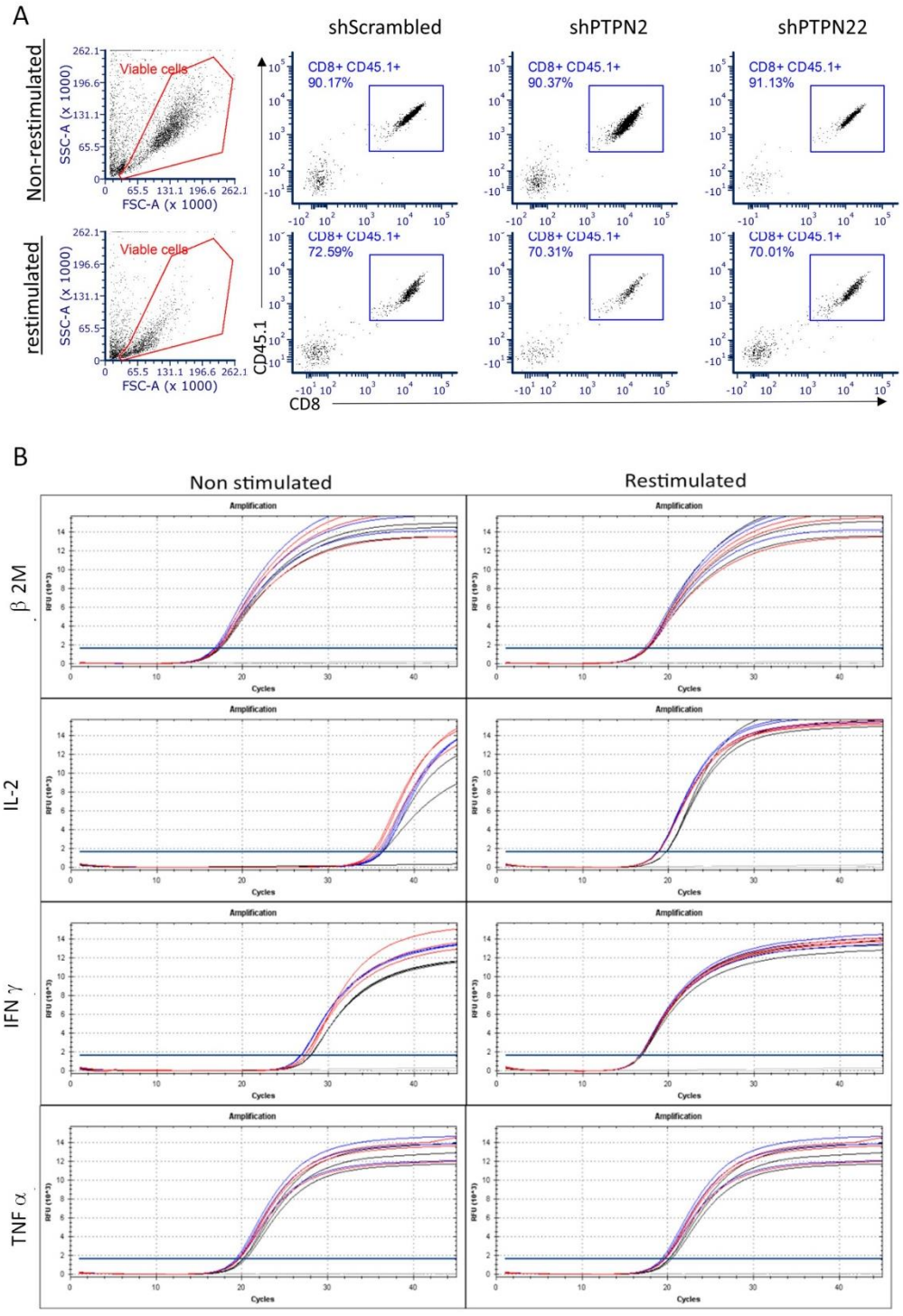
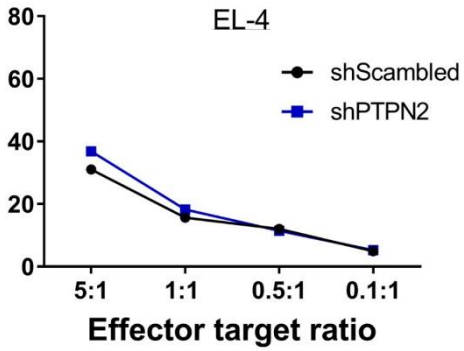


Supplementary figure 5.3: Purification of CD45.1⁺ cells and qPCR analysis of cytokine production in transduced CTLs. Following the protocol established in **Figure 5.7**. A, After the four-hour restimulation, CD45.1+ cells were isolated by positive magnetic selection. Flow cytometry was used to assess purity by measuring the frequency of CD45.1⁺ cells out of viable cells. B, Raw qPCR data displayed in **Figure 5.7A .**



Supplementary figure 5.4: The effect of PTPN2 knockdown on CTLs killing ability. A and B, Naïve OT-I splenocytes were activated and transduced with shPTPN2 or shScrambled following the standard protocol. The transduced cells were purified by FACS (GFP^+) and expanded for five days in IL-2 (10ng/ml) refreshing cytokine daily. Following the expansion, the transduced cells were cultured for four hours with a 1:1 mix of SIIN-pulsed EL4 target cells stained in CFSE with CTV-stained unpulsed EL4 cells Following this incubation, the ratio of target to non-specific cells was determined by flow cytometry by gating on the $CD45.1^-$ population to determine the percent of specific killing. Data are from a single experiment with three replicates.

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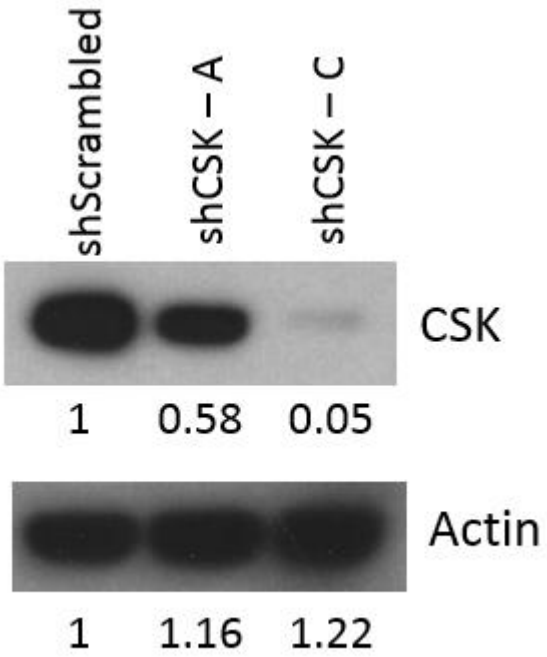
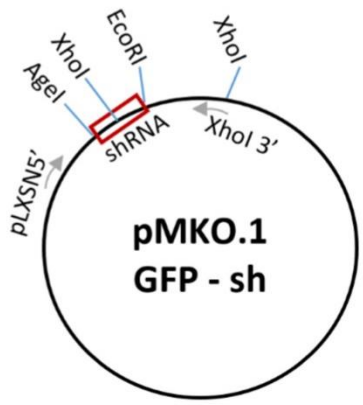


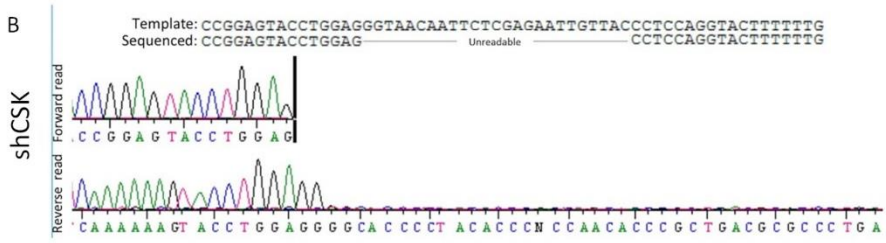
Supplementary figure 5.5: Knockdown of CSK. Continues image of WB described in Figure 5 A.

A



Primer	Location	Read out (58 base pairs)
Forward — pLXSN5'	320bp 5' of AgeI	5' CCGG—21bp sense—CTCGAG—21bp antisense—TTTTTG 3'
Reverse—XhoI 3'	360bp 3' of EcoRI	5' CAAAAA—21bp sense—CTCGAG—21bp antisense —CCGG 3'

B



Supplementary figure 5.6: Sequencing shCSK: A, Schematic detailing forward and reverse sequencing. B, Sequencing data from the forward primer (pLXSN5') and the custom designed reverse primer (XhoI 3').

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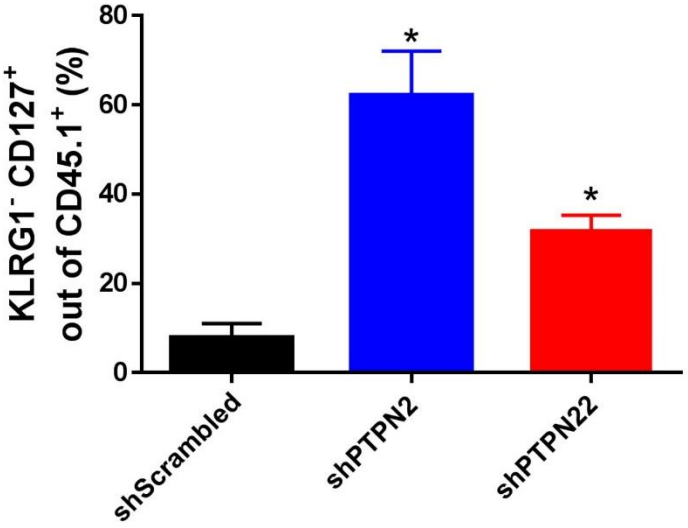

Supplementary figure 5.7: Memory differentiation of transferred cells possessing phosphatase knockdown. Following the protocol detailed in **Figure 5.13,** the phenotype was assessed from peripheral blood samples by flow cytometry on day 15 post transfer into EG7 bearing mice.

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