

Engineering CAR-Mucosal-Associated Invariant T (MAIT) Cells for Cancer Therapy

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Declaration of originality

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Abstract

The advent of chimeric antigen receptor (CAR) technology has revolutionized cancer immunotherapy by empowering T cells to recognize and eliminate tumour cells in a highly specific and efficient manner. In recent years, there has been a heightened interest in harnessing unconventional T cell subsets as potential candidates for CAR-based therapies, aiming to address the existing challenges associated with autologous CAR-T therapy. This thesis investigates the feasibility and functions of mucosal-associated invariant T (MAIT)-based cells as a novel therapeutic strategy for cancer immunotherapy. Primary human MAIT cells from peripheral blood mononuclear cells (PBMCs) were genetically engineered with a third-generation CAR targeting CD19, incorporating a truncated epidermal growth factor receptor (tEGFR) as a tracking marker. Upon CAR activation, CAR-MAIT cells demonstrated high levels of cytotoxicity toward target B cells *in vitro*, comparable to conventional CAR-T cells. The mixed lymphocyte reaction (MLR) assay revealed low alloreactivity of MAIT cells in response to allogeneic stimuli, suggesting their potential for allogeneic therapy. Besides, the study explored optimised manufacturing protocols for generating CAR-MAIT cells, including using retroviral and lentiviral platforms to ensure efficient gene delivery and sustained CAR expression. Additionally, MAIT cell-specific *in vitro* expansion strategies, employing the synthesized major histocompatibility complex (MHC) class-I related protein 1 (MR1) ligand 5-OP-RU and MR1 tetramer-loaded artificial antigen presenting cell (APC) beads, were explored. Investigations into the effects of different cytokine combinations and serum conditions on MAIT cell expansion provided valuable insights into optimising culture conditions for CAR-MAIT cell production. Together, these findings highlight the potential of CAR-MAIT cells as a

promising off-the-shelf approach to cell-based cancer immunotherapy, paving the way for more effective and universally applicable CAR-MAIT cell-based cellular therapies.

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List of Abbreviations

Abbreviation	Definition
ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen presenting cell
AP-1	Activator Protein-1
ALL	Acute lymphoblastic leukaemia
$\alpha\beta$ T cell	Alpha beta T cell
BLCL	B lymphoblastoid cell line
BTk	Bruton's tyrosine kinase
CAR	Chimeric antigen receptor
CDC	Complement-dependent cytotoxicity
CFSE	Carboxyfluorescein succinimidyl ester
CLL	Chronic lymphocytic leukaemia
CRS	Cytokine release syndrome
DAG	Diacylglycerol
DLBCL	Diffuse large B cell lymphoma
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
D10	Completed DMEM with 10% fetal bovine serum
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FL	Follicular lymphoma
$\gamma\delta$ T cell	Gamma-delta T cell

G-Rex	Gas-permeable static cell culture system
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft-versus-host disease
GzmB	Granzyme B
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
iNKT	Invariant natural killer T
ITAMs	Immunoreceptor tyrosine-based activation motif
IL	Interleukin
IFN- γ	Interferon γ
IP3	Inositol trisphosphate
LAT	Linker for activation of T cells
Lck	Lymphocyte-specific protein tyrosine kinase
MAIT	Mucosal associated invariant T cell
MAPK	Mitogen-activated protein kinase
MFI	Mean Fluorescence Intensity
MHC	Major histocompatibility complex
MM	Multiple myeloma
MNC	Mononuclear cells
MR1	MHC-1 related protein 1
MZL	Marginal-zone lymphomas
NFAT	Nuclear Factor of Activated T cell
NF- κ B	Nuclear factor-kappa
NHL	Non-Hodgkin's B cell lymphoma
NTD	Non-transduced control
PBMC	Peripheral blood mononuclear cell
PFA	Paraformaldehyde

PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand 1
PLC- γ 1	Phospholipase C-gamma 1
PIP2	Phosphatidylinositol 4,5 bisphosphate
PKC	Protein kinase C
RPMI 1640	Roswell Park Memorial Institute Medium 1640
r/r disease	Relapsed/refractory disease
R10	Completed RPMI with 10% fetal bovine serum
scFv	Single-chain variable fragment
TAA	Tumour-associated antigen
TCR	T cell receptor
tEGFR	Truncated epidermal growth factor receptor
Tfh	Follicular T helper cell
TGF- β	Transforming growth factor β
TLR	Toll-like receptor
TIM-3	T cell immunoglobulin mucin domain containing protein 3
TNF	Tumour necrosis factor
Th cell	T helper cell
Treg cell	Regulatory T cell
TRUCKs	T cell-redirected universal cytokine-initiated killing
VH	Antibody variable heavy chain
VL	Antibody variable light chain
VSV-G	G glycoprotein of the vesicular stomatitis virus
ZAP-70	Zeta-chain-associated protein kinase 70
ZFN	Zinc-finger nuclease
5-A-RU	5-amino-6-d-ribitylaminouracil
5-OP-RU	5-(2-oxopropylideneamino)-6-d-ribitylaminouracil

Chapter 1. Introduction

1.1. T cell biology

The immune system consists of a complex and intricate network of cells and molecules that work in harmony to defend the body against pathogens, eradicate cancer cells and maintain homeostasis. Among the diverse immune cell populations, T cells emerge as pivotal players in orchestrating immune responses. They possess remarkable specificity and versatility, allowing them to respond to a diverse range of antigens and mount targeted immune responses.

1.1.1. Development and diversity of T cells

T cells can be classified into distinct subsets based on their functions and associated gene or protein expression patterns. Alpha beta ($\alpha\beta$) T cells are a prominent subset of T cells that play a crucial role in adaptive immunity. They derive their name from the presence of $\alpha\beta$ T cell receptors (TCRs) on their cell surface, which enable them to recognize a wide spectrum of peptide antigens presented by Major Histocompatibility Complex (MHC) class I and II molecules. These $\alpha\beta$ T cells are generated through a complex process of T cell development in the thymus, where they undergo positive and negative selection to ensure their specificity and self-tolerance (Klein et al., 2014). Following the selection, the cells differentiate into specialized either $CD4^+$ or $CD8^+$ cells with distinct functions. Once matured, they migrate to peripheral tissues, where they actively participate in immune surveillance and immune responses. $CD4^+$ T cells regulate the activation and differentiation of other immune cells by

producing cytokines and providing help signals, while CD8⁺ T cells recognize and eliminate infected or abnormal cells through direct cytotoxicity (Kumar, Connors & Farber, 2018).

Unlike their conventional counterparts, another subtype of T cells called innate-like T cells express a more limited TCR repertoire and recognize antigens presented by non-classical MHC molecules or antigen-presenting molecules. They exhibit properties that bridge the gap between innate and adaptive immunity (de Araújo et al., 2021; Gao & Williams, 2015). In recent years, significant advancements have led to the identification of two distinct $\alpha\beta$ innate T cell populations in humans: mucosal-associated invariant T (MAIT) cells and invariant natural killer T (iNKT) cells. These two subsets, along with $\gamma\delta$ T cells, comprise the three primary categories of innate T cells (Gao & Williams, 2015). Innate-like T cells often display a memory-like phenotype and can mount potent immune responses without requiring prior exposure to the specific antigen. They play crucial roles in various immune processes, including antimicrobial defence, regulation of autoimmunity, and tumour surveillance (de Araújo et al., 2021). Due to their unique characteristics, innate-like T cells have emerged as a subject of great interest in the field of immunology, driving active research to uncover their functional and clinical significance.

1.1.2. Signaling of T lymphocytes

T cell signaling is a complex and regulated process that plays a crucial role in coordinating the immune response. Upon encountering the antigen presenting cells (APCs), T cells initiate a cascade of intracellular signaling events that ultimately control their activation status, proliferation, and effector functions (Shah et al., 2021).

In general, the transduction of extracellular signals into intracellular responses typically involves the recognition and binding of a ligand to the extracellular portion of a receptor. This interaction triggers the aggregation or conformational alterations of the receptor, facilitating the transmission of external signals to the interior of the cell (Shankaran, Resat & Wiley, 2007). TCR acts as the primary sensor for T cells. Structurally, it is composed of two distinct protein chains, namely α and β chains, or γ and δ chains in the case of gamma-delta ($\gamma\delta$) T cells. Each chain consists of a constant (C) region and a variable (V) region (Shah et al., 2021). The constant region provides structural stability to the receptor complex and allows for proper folding and assembly of the TCR. The variable region is responsible for antigen recognition and its engagement with the MHC molecule triggers a series of phosphorylation events within the TCR complex (Courtney, Lo & Weiss, 2018). As demonstrated by Figure 1.1, upon the interaction of the TCR with the specific antigenic peptide presented by the MHC molecule, the associated CD3 subunits (CD3 γ , CD3 δ , CD3 ϵ , and CD3 ζ) containing immunoreceptor tyrosine-based activation motifs (ITAMs) become phosphorylated by the lymphocyte-specific protein tyrosine kinase (Lck). This leads to the recruitment and activation of various downstream signaling molecules, including zeta-chain-associated protein kinase 70 (ZAP-70) to the phosphorylated ITAMs. Activated ZAP-70 then phosphorylates the adapter proteins, such as the linker for activation of T cells (LAT), which serves as a docking site for

phospholipase C-gamma 1 (PLC- γ 1). The activated PLC- γ 1 then cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 triggers the release of calcium ions from intracellular stores, resulting in an increase in intracellular calcium levels, which is a crucial signaling event in T cell activation. DAG activates multiple downstream proteins, including protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) pathways, which further propagates the downstream signaling cascade. These signaling events ultimately lead to the activation of transcription factors like NFAT (nuclear factor of activated T cells), nuclear factor-kappa (NF- κ B) and AP-1 (activator protein-1), which enter the nucleus and regulate the expression of genes involved in T cell proliferation, cytokine production, and effector functions (Shah et al., 2021; Courtney, Lo & Weiss, 2018).

In terms of T cell activation, the initial interaction between the TCR and the peptide-MHC complex, known as signal 1, is inadequate for complete T cell activation. A second signal, also known as the co-stimulatory signal, is essential for sustaining T cell responses (Azuma, 2019). This co-stimulatory signal involves the interaction of co-stimulatory molecules, such as CD28 on T cells binding to the corresponding ligands CD80/86 expressed on APCs. This engagement provides additional activation and survival signals, preventing the cells from entering a state of anergy, apoptosis, or immune tolerance acquisition (Azuma, 2019; Ogawa & Abe, 2019). Along with signal 1 and 2, T cells receive more instructions in the form of cytokines as a third signal, where cytokines secreted by APCs and other immune cells bind to their corresponding receptors on T cells. These cytokines modulate T cell differentiation, survival, and functional specialization, influencing the outcome of the immune response cells (Curtsinger & Mescher, 2010) (Figure 1.1). The integration of these three signals is critical for optimal T cell activation and the subsequent development of effective immune responses.

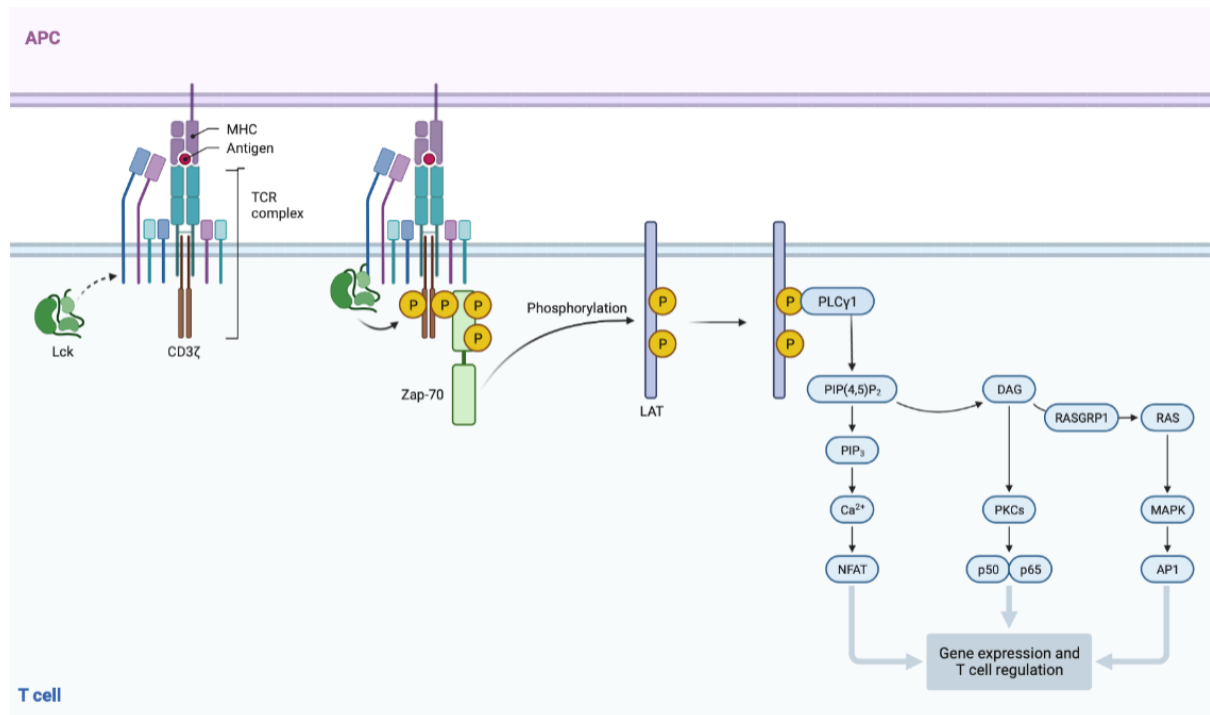


Figure 1.1. T cell receptor signalling.

The interaction between a TCR and peptide-MHC initiates a cascade of events that modulate T cell signaling. Upon antigen recognition, the intracellular domains of CD3 undergo phosphorylation of ITAMs by Lck. This phosphorylation event facilitates the recruitment and subsequent phosphorylation of ZAP-70. ZAP-70, in turn, phosphorylates key adaptor molecules such as LAT. LAT then serves as the scaffold for assembling PLC-γ. Activated PLC-γ cleaves PIP₂ into IP₃ and DAG. IP₃ induces calcium release from intracellular stores, while DAG activates PKC. The combined signals from IP₃, DAG, and calcium lead to the translocation of NFAT, NF-κB p50/65 and AP-1 transcription factors into the nucleus. Once in the nucleus, these transcription factors regulate the expression of multiple genes involved in T cell proliferation and differentiation, contributing to the overall activation of T cells. Figure adapted from Azuma, 2019; Ogawa & Abe, 2019, and modified using BioRender.

1.1.3. T cell roles in cancers

T cells, as a part of the adaptive immunity, are responsible for patrolling and distinguishing the foreign and abnormal entities, including the pathogenic invaders and malignant cells. Upon the specific recognition of the tumour-associated antigens (TAAs), multiple activation signalling cascades are triggered to induce the effector T cell activities, including proliferation, differentiation, cytokine production and cytotoxicity against the malignant cells (Waldman, Fritz & Lenardo, 2020). Effector T cells, including cytotoxic CD8⁺ T cells and helper CD4⁺ T cells, are involved in antitumour responses. Cytotoxic CD8⁺ T cells can directly recognize the tumour-specific antigens presented on the surface of cancer cells by MHC-I molecules and release cytotoxic molecules, such as perforin and granzymes, which induce apoptosis in the target cancer cells. In addition, CD8⁺ T cells express the Fas ligand. Upon interaction with the corresponding Fas receptors on the target cells, it triggers a cascade of caspases and endonuclease activities, leading to the fragmentation of DNA within the target cells (Raskov et al., 2021). On the other hand, helper CD4⁺ T cells are activated via MHC class II molecules and contribute to antitumour immunity by providing immune support (Haabeth et al., 2014). Based on the cytokines they secreted, helper CD4⁺ T cells are categorized into distinct subsets, including Th1, Th2, Th17, Treg and Tfh cells, each with specialized functions in modulating the immune response. For instance, Th1 cells secrete interferon-gamma (IFN- γ) and tumour necrosis factor (TNF) to maximize the killing efficacy of macrophages and enhance the proliferation of cytotoxic CD8⁺ T cells, while Th2 produce interleukin (IL)-4 to stimulate promote B cell-associated humoral immune responses (Golubovskaya & Wu, 2016; Ahmed et al., 2023). The coordinated efforts of cytotoxic CD8⁺ T cells and helper CD4⁺ T cells contribute to the overall antitumour immune responses.

However, cancer cells often evade or suppress the immune system, enabling their growth and spread. The intricate interplay between cancer cells and T cells can give rise to various mechanisms of immune evasion and escape. For instance, the tumour-released molecules like IL-10 and transforming growth factor β (TGF- β) exert immunosuppressive effects, dampening anti-tumour responses. Foxp3⁺ regulatory T (Treg) cells recruited to the tumour microenvironment further attenuate the functional activities of effector T cells (Labani-Motlagh, Ashja-Mahdavi & Loskog, 2020). Besides, cancer cells can modulate the expression of surface molecules to impede immune surveillance and activity. Alterations in MHC-I expression or deficiencies in antigen processing machinery make cancer cells less antigenic, evading recognition by patrolling T cells. Additionally, tumour cells can induce immune tolerance, countering anti-tumour immune responses. Upregulation of programmed death ligand 1 (PD-L1) on the surface of cancer cells plays a pivotal role in modulating immune responses by engaging with programmed cell death protein 1 (PD-1) receptors on T cells. This interaction induces T cell anergy and depletion, thereby impairing the immune system's ability to mount an effective anti-tumour response (Labani-Motlagh, Ashja-Mahdavi & Loskog, 2020; Vinay et al., 2015). Patients with advanced tumours are often in the dilemma of inadequate tumour-infiltrating lymphocytes and compromised T cell activity (Majzner & Mackall, 2019; Arcangeli et al., 2020). Hence, there is a pressing need to devise effective strategies for expanding tumour-reactive T cells and enhancing their activity within tumour-derived immunosuppression.

1.2. Cancer immunotherapy

For years, surgery, radiation and chemotherapy form the foundation of the standard cancer treatment. However, the landscape has evolved significantly in recent decades with the emergence of two ground-breaking advancements: chimeric antigen receptor (CAR) T-based immunotherapy and the introduction of checkpoint inhibitors, particularly PD-1/PD-L1 blockade. These developments have propelled immunotherapy to the forefront of cancer treatment, offering a paradigm shift by leveraging the body's own immune system to combat cancer cells while preserving healthy tissues (Hosseinkhani et al., 2020). This transformative strategy marks a significant departure from conventional cancer therapeutics.

1.2.1. Chimeric antigen receptor

CAR-T cell therapy represents a pioneering immunotherapeutic approach that has revolutionized the landscape of cancer treatment (Alnefaie et al., 2022). The basic principle of CAR is to redirect the specificity of the immune cells by employing a high-affinity antigen recognition domain derived from a monoclonal antibody and activating the cytolytic functions of immune cells through intracellular signalling domains upon antigen recognition (Mchayleh et al., 2019). By genetically modifying T cells to express CAR on the cell surface, it augments the T cells' inherent capacity to effectively recognize the specific cancer-associated antigens and enhances their efficacy in combating the malignancies.

The production of CAR-T cells follows a well-defined workflow comprising several crucial steps to ensure the optimal therapeutic efficacy (Blache et al., 2022) (Figure 1.2). Initially, human peripheral blood mononuclear cells (PBMCs) are collected from patient or donor blood. The

desired immune cells, usually T cells, are isolated for subsequent genetic modification. The virus-based transduction methods, employing retroviral and lentiviral vectors, have emerged as one of the robust tools for stable and efficient modification of CAR-T cells, owing to their remarkable transduction efficiency and safety demonstrated in numerous clinical trials (Wang & Rivière, 2016; Blache et al., 2022). Later, these modified T cells undergo *in vitro* expansion to reach the satisfactory level for treatment. Following infusion, the tumour-specific CAR-T cells execute their therapeutic function by actively targeting and eliminating malignancies. This powerful immunotherapeutic approach harnesses the natural cytotoxicity of T cells and redirects their specificity towards tumour-associated antigens, resulting in potent and targeted anti-cancer activity.

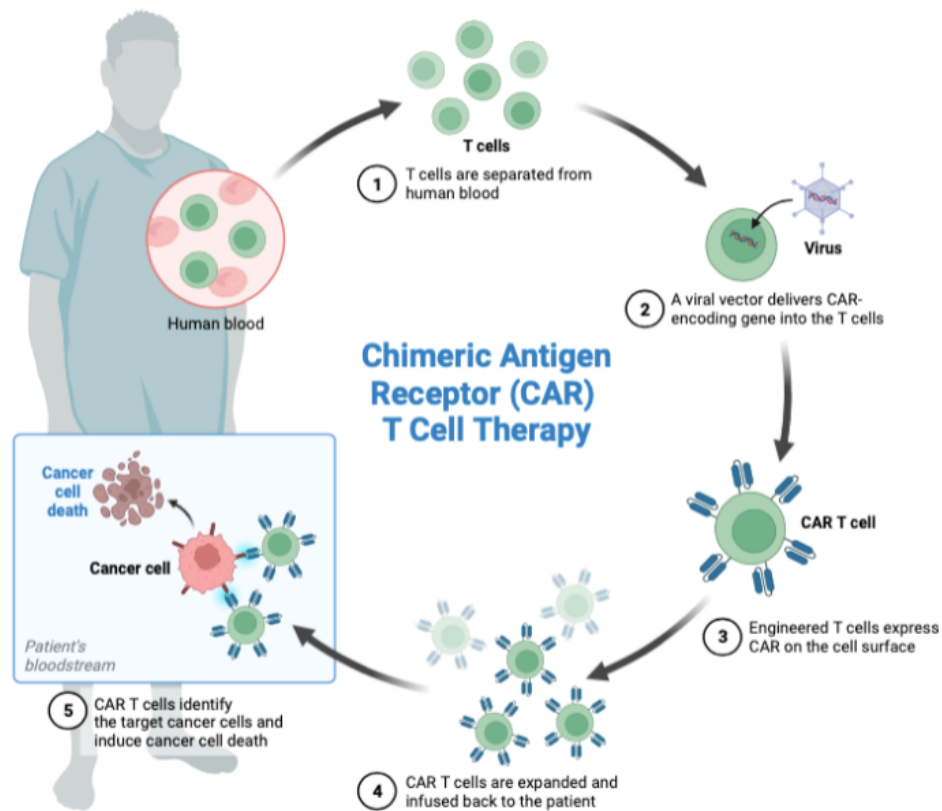


Figure 1.2. Overview of CAR-T manufacture and therapy.

(1) PBMCs are collected from human blood and processed to isolate T cells. (2) Delivery of CAR transgene via viral vectors allows expression of the desired CAR on T cell surface. (3) Modified T cells with CAR are endowed with redirected and enhanced tumour recognition capabilities. (4) CAR-T cells undergo clinical-scale expansion *in vitro* and infused back to the patient. (5) CAR-T cells selectively recognize the target cancer cells, subsequently triggering a cascade of cellular events that induce the cancer cell death and eradicate the malignant entities within the patient's body. Figure adapted from Blache et al., 2022 and modified using BioRender.

1.2.2. Structures and generations of CAR

Since the introduction of CAR technology, the designs of CAR constructs have undergone progressive refinements. These successive generations of CAR constructs have been characterized by continuous advancements, incorporating novel features and modifications aimed at enhancing the therapeutic potential of CAR-T cell therapy (Zhang et al., 2017; Tokarew et al., 2019). The typical CAR, also known as 1st generation, is composed of an extracellular single chain variable fragment (scFv) for MHC-independent antigen recognition, a transmembrane domain for the stability of CAR structure and an intercellular CD3 ζ signalling domain for T cell activation and functions (Tokarew et al., 2019) (Figure 1.3). In general, an scFv consists of a variable light chain (VL) and a variable heavy chain (VH) derived from an antibody, linked together by a flexible peptide linker to enhance its adaptability. This design ensures that the scFv retains the specificity of the original monoclonal antibody (Ahmad et al., 2012). When incorporated into CAR-T cells, the scFv equips the modified cells with the ability to recognize a broad spectrum of antigens, including proteins, glycolipids, and carbohydrates (Alnefaie et al., 2022). Unlike conventional $\alpha\beta$ TCRs, CAR-T cells are not constrained by MHC molecules. Therefore, CAR-T cell engineering overcomes the limitations imposed by MHC downregulation and alteration in cancer cells. Besides, the affinity between the scFv and target proteins is comparatively higher when compared to the natural affinity between TCRs and peptide-MHC complexes (Mao, Kong & He, 2022). This enhanced affinity has the potential to augment the specificity and sensitivity of CAR-T cells in their recognition and targeting capabilities. In terms of the intracellular signaling domain, it has been established that the CD3 ζ domain alone is capable of effectively initiating signaling events required for the activation of host T cells. Therefore, the CD3 ζ motif is frequently selected as the signaling component in the design of CAR constructs (Brocker, 2000). However, the

absence of the signal 2 mediated by co-stimulation has been identified as the primary factor restricting the *in vivo* expansion and persistence of 1st generation CAR-T cells (Brocker & Karjalainen, 1995; Brocker, 2000).

To enhance T cell proliferation and activity, advancements in CAR design involve incorporating additional costimulatory and cytokine production units into the CAR intracellular domain (Figure 1.3). CD28 or/and 4-1BB domains are the most widely applied costimulatory units found in the 2nd and 3rd generation of CAR constructs that can enhance sustained T cell proliferation and alleviate exhaustion. Besides, costimulatory molecules OX-40, ICOS and CD27 have been shown to facilitate and improve CAR-T cell activity and persistence *in vivo* (Mohanty et al., 2019; Stock, Schmitt & Sellner, 2019). Clinical studies have revealed that patients with relapsed/refractory (r/r) disease and B cell malignancy that accepted 2nd generation CAR-T cell therapy showed superior clinical outcomes compared to 1st generation, attributed to the enhanced functions of CAR-T cells (Al-Mansour, Al-Foheidi & Ibrahim, 2020). The 3rd generation of CAR incorporates two costimulatory domains attached to the signalling domain as a further signal enhancement. Clinical trials involving 3rd generation CAR-T cell products have demonstrated a remarkably favourable safety profile, while simultaneously yielding enhanced clinical outcomes in patients with B cell malignancies (Shadman et al., 2020; Enblad et al., 2018).

Early development of CAR designs primarily emphasized the enhancement of T cell-mediated anti-cancer responses. Development of 4th generation CAR has introduced a novel concept called T cell-redirected universal cytokine-initiated killing (TRUCKs) (Figure 1.3). This approach is aimed at remodelling the immunosuppressive tumour microenvironment, transforming it

into an immunologically supportive milieu that fosters robust anti-tumour immune responses. It involves incorporating immune modulators, including proinflammatory cytokines and costimulatory ligands, into the CAR construct through additional genetic modifications to the intracellular signaling domain (Stock, Schmitt & Sellner, 2019). The attempt of incorporating proinflammatory cytokines IL-7 and CCL-19 into the CAR construct has demonstrated superior cell proliferation, elevated cytotoxicity, and infiltration in the treatment of r/r multiple myeloma compared to 2nd generation CAR-T cells (NCT03778346) (Duan et al., 2021). However, this strategy requires careful regulation in the cytokine release amount to avoid cytokine storm.

It is worth noting that the majority of existing CAR systems typically adhere to fixed designs, characterized by a consistent recognition domain and static signaling domains. Yet, these inherent design constraints present obstacles in adequately combating cancer relapse linked to antigen-negative tumours and addressing potential therapy-related toxicities (Labanieh & Mackall, 2023). First of all, the emergence of antigen escape introduces a notable hurdle in CAR-T cell therapy, as the rigid structure of the CAR cannot be easily modified. Consequently, the only viable option to address this issue is through the re-infusion of re-engineered CAR-T cells into patients, a process that is both resource-intensive and time-consuming, thereby adding to the complexity and cost of the therapy (Liu, Zhao & Song, 2019). In addition, managing T cell toxicities associated with this fixed design becomes a significant concern due to limited controllability. Simultaneous activation of signaling domains at a pre-determined level occurs upon identification of the antigen by the CAR (Minutolo et al., 2020). To overcome these limitations, a novel CAR strategy, employing a split, universal, and programmable CAR construct design, has emerged. Intriguingly, Minutolo *et al.* and Cho *et al.*

have pioneered a novel CAR system constituting two components: one universal zip-receptor paired with an antigen-recognising scFv zip-adaptor (Minutolo et al., 2020; Cho, Collins & Wong, 2018) (Figure 1.3). The special 'lock and key' structure of the leucine zipper allows targeting multiple antigens with a single cell product via adjustable pairing with various tumour-targeting ligands. Besides, competitive leucine zippers act as the OFF switch to control T cell activity and mitigate associated toxicities when required (Cho, Collins & Wong, 2018). The so-called SpyTag-SpyCatcher platform serves as an advantageous strategy for the development of split CAR systems. This approach is based on the natural interaction between the small peptide sequence, SpyTag, and its protein counterpart, SpyCatcher. SpyTag is a short peptide (13 amino acids) that can covalently bind to the reactive surface of SpyCatcher through an isopeptide bond formation (Minutolo et al., 2020). In the context of CAR-T cell therapy, SpyCatcher is linked with the intracellular costimulatory and CD3 ζ signaling domains and embedded on the T cell surface. Concurrently, the specific SpyTag is linked to an scFv and forms a covalent bond with the SpyCatcher. Applying the SpyTag-SpyCatcher system in CAR-T cell manufacturing enables precise modulation of T cell killing functions in a dose-dependent manner, resulting in the suppression of tumour growth. This pioneering technique offers a promising avenue for the reconfiguration of T cells and underscores the significance of pairing T cells with appropriate CAR constructs to achieve the desired therapeutic objectives (Liu et al., 2020; Minutolo et al., 2020).

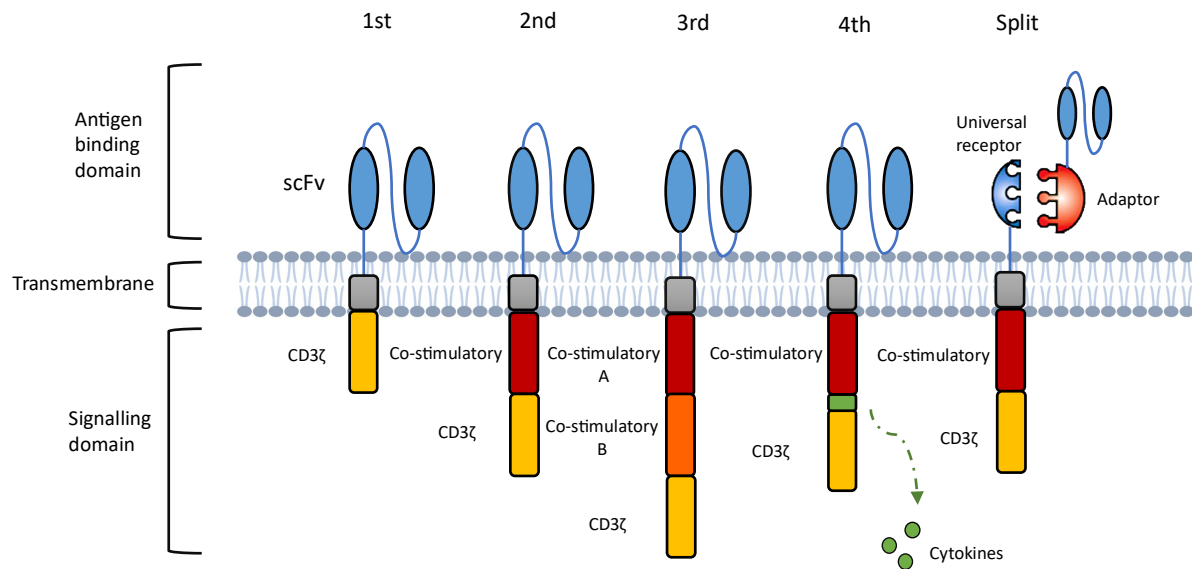


Figure 1.3. CAR structures and generations.

The 1st generation of CAR contains an extracellular antigen binding domain and an intracellular signalling domain while the 2nd and 3rd possess one or more co-stimulatory domains (e.g. CD28, 4-1BB, OX-40, ICOS, CD27) to facilitate T cell activity. The 4th generation CAR (TRUCK) can express immune modifiers (e.g. proinflammatory cytokines) via an extra genetic modification on the CAR construct. The novel split CAR system contains two 'lock and key' components, one universal receptor and a specific adaptor (e.g. SpyTag-SpyCatcher system), for adjustable scFv pairing. Figure adapted from Tokarew et al., 2019; Minutolo et al., 2020 and modified using BioRender.

1.2.3. Current application of CAR-T cell therapy

CAR-T cell therapy has demonstrated remarkable therapeutic outcomes in multiple clinical trials for hematologic malignancies. Targeting CD19 with CAR-T cells has yielded impressive complete response rates ranging from 50% to 90% in patients with lymphocytic leukaemia (Labanieh, Majzner & Mackall, 2018). The unprecedented success of CAR-T cell-based anticancer therapy has gained approvals from regulatory agencies such as the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Table 1.1 presents an overview of FDA-approved CAR T cell-based therapies. The FDA has granted authorization for the utilization of six autologous CAR-T products derived from patients' autologous blood cells to treat B cell malignancies. Among these, four are the T cell products that exhibit specificity towards CD19, namely Kymriah® (Tisagenlecleucel) (Maude et al., 2018), which has demonstrated efficacy in patients with r/r acute lymphoblastic leukaemia (ALL); Yescarta® (Axicabtagene Ciloleucel) (Neelapu et al., 2017) and Breyanzi® (Lisocabtagene Maraleucel) (FDA, 2021b), both approved for the treatment of r/r diffuse large B cell lymphoma (DLBCL); and Tecartus® (Brexucabtagene Autoleucel) (FDA, 2020), authorized for patients with r/r mantle cell lymphoma (MCL). Abecma® (Idecabtagene Vicleuce) represents the pioneering BCMA-directed CAR T-cell therapy approved for commercial use in treating patients with multiple myeloma (MM) (FDA, 2021a). Additionally, Carvykti® (Ciltacabtagene Autoleucel), another BCMA-targeting therapy, obtained approval in 2022 for the treatment of relapsed or refractory MM (FDA, 2023). The CAR-T cell-based approach, with its enhanced tumour specificity, durable persistence, and reduced off-target effects, holds immeasurable potential as a novel cancer treatment regimen.

Table 1.1. Overview of representative CAR-T cell products for cancers

Target Antigen	Diagnosis	Company/ Institution	Approval/ Phase	References
CD19	B-cell Acute Lymphoblastic Leukemia (B-ALL), Diffuse Large B-cell Lymphoma (DLBCL)	Novartis	FDA-approved Kymriah® (Tisagenlecleucel)	Maude et al., 2018
CD19	Large B-cell Lymphoma, Diffuse Large B-cell Lymphoma (DLBCL), Follicular Lymphoma (FL)	Kite Pharma	FDA-approved Yescarta® (Axicabtagene Ciloleucel)	Neelapu et al., 2017
CD19	Large B-cell Lymphoma, Diffuse Large B-cell Lymphoma (DLBCL)	Juno Therapeutics	FDA-approved Breyanzi® (Lisocabtagene Maraleucel)	FDA, 2021b
CD19	Mantle Cell Lymphoma (MCL)	Kite Pharma	FDA-approved Tecartus® (Brexucabtagene Autoleucel)	FDA, 2020
BCMA	Multiple Myeloma (MM)	Celgene Corporation	FDA-approved Abecma® (Idecabtagene Vicleucel)	FDA, 2021a
BCMA	Multiple Myeloma (MM)	Janssen Biotech	FDA-approved Carvykti® (Ciltacabtagene Autoleucel)	FDA, 2023

1.2.4. Current challenges of autologous CAR therapy

Advances in CAR-T cell therapy have revolutionized the field of cancer treatment, offering remarkable efficacy and durable effects for patients. Despite its promising potential, this innovative approach encounters several challenges that hinder its broad application and optimal effectiveness. One primary obstacle lies in the complex and time-consuming process of manufacturing the personalized CAR-T cells for each patient. Currently, the commercially available CAR-T therapies utilize conventional CD8⁺ effector T cells derived from the patient's own blood as the backbone for CARs. This patient-derived CAR-T cell manufacturing requires approximately 20 days to generate CAR-T cells, followed by additional time for product transportation (Vormittag et al., 2018). Acquiring a substantial quantity of high-quality T cells from patients with advanced diseases who have undergone prior chemotherapies poses a significant challenge. Patients with cancer may encounter manufacturing failures due to lymphocytopenia and poor cell fitness, posing challenges to quality control and reproducibility of stable clinical-grade cell products. This personalized approach is resource-intensive, time-consuming, and costly, hindering scalability and the treatment of a large number of patients. The potential of off-target toxicity and adverse events induced by CAR-T cells poses safety concerns. If the CAR-T cells target antigens that are also expressed on normal cells, it would lead to unintended immune responses and tissue damage. Cytokine release syndrome (CRS) and neurotoxicity are the well-known complications associated with CAR-T cell therapy, requiring careful management and monitoring (Mohanty et al., 2019).

1.2.5. Potential of allogeneic CAR therapy

Ongoing effort to establish "off-the-shelf" cell products represent a compelling alternative strategy aimed at enhancing the accessibility of CAR therapy for patients. Utilizing the allogeneic T cells derived from healthy donors offers a host of advantages over the autologous approaches. First of all, allogeneic cells serve as a more standardized starting material for the therapy, thereby facilitating more predictable manufacturing processes and enhancing the performance of the resulting cell product. Allogeneic CAR therapy can leverage the use of healthy donor T cells with superior quality and proliferative capacity, potentially enhancing the efficacy and persistence of CAR-T cells. Second, allogeneic therapy holds the potential to offer ready-to-use immunotherapeutic products without the need for individualized manufacturing, making allogeneic CAR therapy more accessible and suitable for patients who require urgent treatment. A single manufacturing run can provide treatment for multiple patients or multiple doses for individual patients. By scaling up production and establishing a pool or bank of manufactured CAR cells sourced from healthy donors, the cost per patient could decrease while ensuring broader accessibility to the therapeutic product (Caldwell, Gottschalk & Talleur, 2021).

However, the major concerns of allogeneic therapy are the risks of life-threatening graft-versus-host disease (GVHD) or rejection by the host immune system. Recognition and interaction between TCR and alloantigen presented by human leukocyte antigens (HLAs) is the key determinant of T cell alloreactivity (Brown & Byersdorfer, 2020). Therefore, in the allogeneic cell therapy, the native TCR of the infused CAR-T cells from the donor may recognize the recipient tissues as foreign, eventually triggering tissue cell death and leading to organ failure in some severe cases. On the other side, the host immune cells may also treat

the donor cells as invaders and eradicate these infused cells (Sanber, Savani & Jain, 2021). Different approaches have been proposed to address these issues. Disruption of either α or β constant chain of TCR is one of the strategies that can cause failure to form fully functional TCR heterodimers on the cell surface (Lo Presti et al., 2020). Early attempts used Zinc-finger nuclease (ZFN) as a genetic scissor to knock out $\alpha\beta$ TCR-related genes and the modified anti-CD19 T cells showed sufficient efficacy to combat B-lineage malignancies without triggering clinically significant GVHD (Torikai et al., 2012). Intriguingly, Eyquem *et al.* introduced a brilliant strategy to direct the CD19-specific CAR to the constant α TCR locus by CRISPR/Cas9, allowing simultaneous TCR inactivation in common with CAR insertion. Allogeneic anti-CD19 CAR-T cells generated with this method demonstrated enhanced anticancer potency and delayed exhaustion in the murine model with ALL (Eyquem et al., 2017). Gene editing has emerged as the leading technique for developing the allogeneic CAR treatment platform. As gene editing tools progress, this procedure has the potential to significantly reduce the dangers and drawbacks connected with the use of allogeneic cells. However, at the present stage, the current gene editing techniques only achieve partial depletion of $\alpha\beta$ TCR in most of the trials and the remaining $\alpha\beta$ -positive ones may still trigger the unwanted toxicity against the host (Caldwell, Gottschalk & Talleur, 2021). In addition, the short-term and long-term safety of these gene altered products in patients remains to be investigated.

Beyond the genetic editing methods, an alternative strategy has been proposed to achieve allogeneic cell therapy. Ongoing efforts are now dedicated to investigating the therapeutic potential of diverse T cell populations as the CAR backbone, which not only holds promise for further expanding the repertoire of CAR-T cell therapy but also addressing the limitations associated with conventional T cells in the context of cancer immunotherapy. Innate-like T

cells, such as $\gamma\delta$ T cells and iNKT cells, have surfaced as captivating candidates for this purpose. $\gamma\delta$ T cells, characterized by their MHC-independent antigen recognition, exhibit minimal alloreactivity and a reduced risk of GVHD, providing a distinct advantage for immunotherapy (Legut, Cole & Sewell, 2015). While iNKT cells possess innate-like properties that can swiftly produce a variety of cytokines upon activation (de Araújo et al., 2021). Both cell types have demonstrated promising anti-tumour effects in preclinical models and early-phase clinical trials (de Araújo et al., 2021; Poels et al., 2021; Capsomidis et al., 2018). Current research is expanding to encompass the other T cell subsets that express unconventional TCRs to further diversify the CAR-T cell landscape. Within this expanding landscape, mucosal-associated invariant T (MAIT) cells, as another member of the innate-like T cell family, have stepped into the spotlight. Despite their relatively youthful presence in research, MAIT cell has emerged as a subject of significance in autoimmunity, infectious disorders, and malignancy (Hinks & Zhang, 2020; Provine & Klenerman, 2020; Lukasik, Elewaut & Venken, 2020). Ongoing studies are unravelling their complex biology and exploring their roles in various diseases. This has sparked our research interest in exploring the therapeutic potential of MAIT cells in immunotherapy.

1.3. MAIT cells as novel candidates for immunotherapy

The investigation of MAIT cells is a relatively new chapter in the research of immune cells. Dating back to their discovery in the early 1990s, they were initially identified as a distinct T cell population expressing an invariant TCR α -chain (V α 7.2–J α 33 in humans and V α 19–J α 33 in mice). The term MAIT cell has been established due to the relative enrichment of these T cells within mucosal tissues and liver of both humans and mice (Porcelli et al., 1993; Tilloy et al., 1999). It was until the 2000s, the investigations began to reveal their unique biology and precise function (Treiner et al., 2003). Since then, research efforts have escalated, uncovering the diverse functional repertoire of MAIT cells, their contribution to immune homeostasis, and their involvement in a spectrum of diseases (Le Bourhis et al., 2010; Gold et al., 2010). As the exploration of MAIT cells continues to expand, their distinct properties and potential therapeutic applications have emerged as an exciting area in immunotherapy research.

1.3.1. Characteristics of MAIT cells

MAIT cells account for 0.1% to 10% of total T cells in human blood (Gherardin et al., 2018a). The frequency of MAIT cells were the lowest in cord blood, followed by a gradual rise until youth, and subsequent decline from youth to the elderly phase (Chen et al., 2019). As their name indicated, they obtain a natural tropism for the mucosal sites, lung, and liver, making up 30 to 50% of the tissue resident T cells (Dusseaux et al., 2011; Kurioka et al., 2016). Unlike the conventional T cells, MAIT cells are restricted to the recognition of non-peptide metabolite antigens presented by the non-polymorphic MHC class-1 related protein 1 (MR1) rather than the typical MHC molecules. MR1 possesses a distinctive feature in its high degree of evolutionary conservation observed in the α 1 and α 2 domains, which correspond to the

peptide-binding regions found in classical MHC class I molecules. This conservation demonstrates approximately 90% amino acid identity between human and mouse variants of MR1 (Riegert, Wanner & Bahram, 1998; Tsukamoto et al., 2013). The invariant TCR of MAIT cells is composed of a unique V α 7.2-J α 33 α chain predominantly paired with V β 2 or V β 13 β chain that distinguishes them from the conventional T cells (Hinks & Zhang, 2020). Given the restriction to non-polymorphic antigen-presenting molecules, their TCRs offer what can be termed as pan-recipient compatibility, thus mitigating the risk of GVHD. This property suggests the potential for transferring the cells to every patient, circumventing the need to isolate HLA-matched tumour-specific TCRs for individual recipients (Vacchini et al., 2020). Owing to these special features, MAIT cells have the potential to overcome the limitations of extensive HLA polymorphism. In other words, they may be suitable for allogeneic cell therapy and are less likely to cause adverse side effects. Human MAIT cells are also characterized by expression of elevated levels of the C-type lectin CD161 and the IL-18R α (Figure 1.4). These markers are often used in combinations to define MAIT cells (Billerbeck et al., 2010; Ussher et al., 2014; Fergusson et al., 2014). Recently, since the successful synthesis of long-lasting and kinetically stable 5-OP-RU loaded MR1 tetramer products, this approach has emerged as the definitive benchmark for the identification of MAIT cells based on their MR1-restricted specificity (Mak et al., 2017a; Gherardin et al., 2018a).

Human MAIT cells can be further subgrouped based on their differential expression of CD4 and CD8 co-receptors. The predominant subset among MAIT cells is the CD4⁻ CD8⁺ subset, accounting for roughly 80% of the MAIT cells found in the circulation of healthy adults. The CD4⁻ CD8⁻ (double negative) subset makes up around 15% of the total MAIT cell population, while the CD4⁺ CD8⁻ and CD4⁺ CD8⁺ (double positive) subsets collectively constitute less than

10% of the total MAIT cells in blood (Chen et al., 2019; Gherardin et al., 2018a). It has been reported that CD8⁺ MAIT cells and the double negative MAIT cells are functionally distinct but developmentally related (Dias et al., 2018). Among MAIT cell subtypes, CD8⁺ MAIT cells exhibit elevated basal expression levels of IL-12R and IL-18R and heightened functional responses to stimuli compared to their counterparts. They demonstrate elevated secretion of cytolytic molecules including IFN- γ , TNF, and granzyme B, while the double negative MAIT cells demonstrate an elevated production of IL-17 (Dias et al., 2018). The functional variations and post-stimulatory effects of each subset remain to be fully elucidated, but it is likely that each subset adds its distinct hue to the overall immunological context. It is also noteworthy that the majority of circulating MAIT cells exhibit a tissue-homing effector phenotype with memory-like characteristics, with expression of CD45RO⁺, CD45RA⁻, CCR5⁺, CCR6⁺, CXCR6⁺, CCR7⁻ and CD62L⁻ (Figure 1.4) (Dusseaux et al., 2011; Gherardin et al., 2018a). The expression of these homing receptors enables MAIT cells to migrate to the liver, intestines, and lungs (Chen et al., 2019). Additionally, they are marked by notable expression of CD28 and the IL-7R α chain CD127, while levels of the costimulatory molecule CD27 and the adhesion molecule CD56 exhibit variability (Gherardin et al., 2018a). The unique characteristics of MAIT cells have made them an intriguing subject of study in the field of immunology. Investigating their distinct functions and implications offers the potential to deepen our knowledge of MAIT cell biology, leading to more targeted therapeutic approaches in the complex landscape of human health and disease.

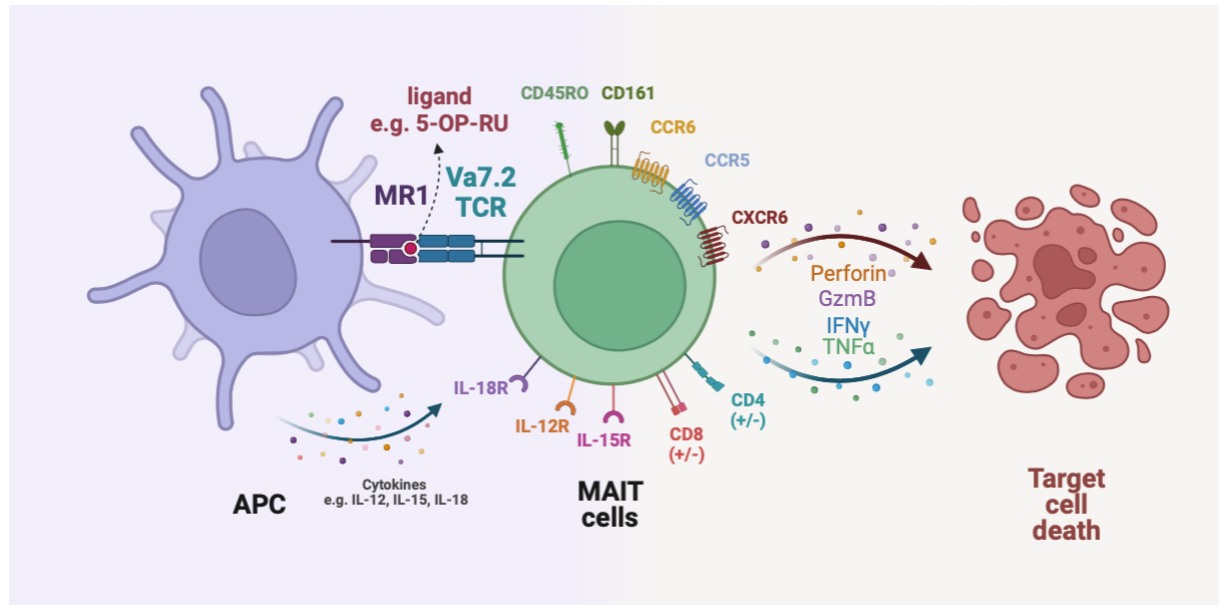


Figure 1.4. Characteristics and functional responses of MAIT cells.

A representation of known biologically significant features found on MAIT cells. MAIT cells have the distinctive Va7.2 TCR to recognize the ligand (e.g. 5-OP-RU) presented by MR1 molecule. They also express the cytokine receptors (e.g. IL-12R, IL-15R, IL-18R) and chemokine receptors (e.g. CCR5, CCR6, CXCR6). In conjunction with Va7.2 TCR or IL-18R, CD161 serves as a key marker for identifying MAIT cells. MAIT cells can be divided into subsets based on the expression of CD8 (+/-) and CD4 (+/-). Most of the circulating MAIT cells are CD45RO⁺ CD45RA⁻. MAIT cells execute their cytolytic functions by secreting granzymes (GzmB) and perforins to kill the target cells through apoptosis. They also engage in the pro-inflammatory response by releasing a spectrum of cytokines, including IFN-γ, and TNF-α. Figure adapted from Gherardin et al., 2018a; Hinks & Zhang, 2020 and modified using BioRender.

1.3.2. MAIT cell activation and functional responses

Like the conventional T cells, MAIT cells follow a well-defined developmental pathway, initiated within the thymus, and undergo extrathymic maturation. During this process, they acquire functional properties that enable them to integrate into various tissues throughout the body. MAIT cells can be activated by the specific intermediates in the vitamin B synthesis pathway, particularly from riboflavin (vitamin B2) and folic acid (vitamin B9) via the interaction with the MR1 molecule. To date, the metabolites derived from the riboflavin biosynthesis pathway of microorganisms, 5-A-RU (5-amino-6-d-ribitylaminouracil) and 5-OP-RU [5-(2-oxopropylideneamino)-6-d-ribitylaminouracil], have been identified as the most potent stimulatory ligands for MAIT cells (Provine & Klenerman, 2020; Corbett et al., 2014). Riboflavin is synthesized a broad range of bacteria, from *Escherichia coli* to *Mycobacterium tuberculosis* (Howson, Salio & Cerundolo, 2015). The pathway begins with 5-A-RU as a crucial intermediate in riboflavin biosynthesis by bacteria, and the non-enzymatic Schiff base condensation of 5-A-RU with α -dicarbonyl compounds, specifically glyoxal or methylglyoxal, leads to the formation of 5-OP-RU. Importantly, 5-OP-RU can persist and be captured through a covalent bond with Lys43 of the MR1 molecule. This covalent bond between 5-OP-RU and MR1 stabilizes the antigen and anchors it for subsequent presentation to the TCR of MAIT cells, thereby activating and eliciting responses (Figure 1.5) (Mak et al., 2017; Tang et al., 2020).

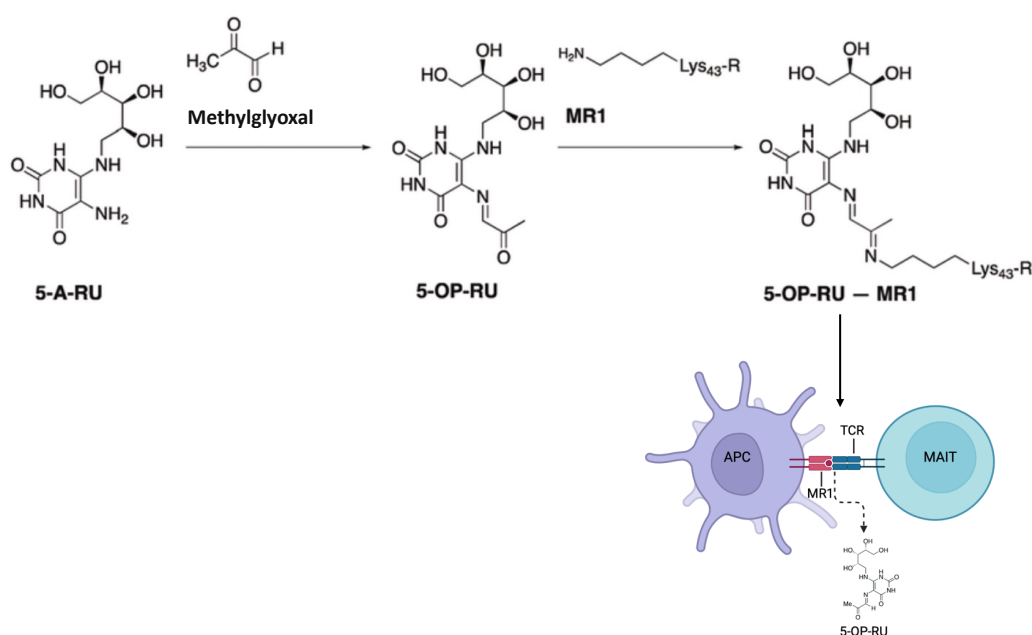


Figure 1.5. Formation of 5-OP-RU.

5-OP-RU, a potent MAIT cell activator, is formed through the condensation of microbial 5-A-RU with methylglyoxal. The resulting 5-OP-RU is covalently bonded to MR1 for subsequent presentation to MAIT cells, activating MAIT cells and facilitating host-pathogen interactions. Figure adapted from Mak et al., 2017; Tang et al., 2020 and modified using BioRender.

Intriguingly, MAIT cells can be quickly activated even in absence of TCR stimulation, owing to their elevated expression of diverse cytokine and chemokine receptors. Upon exposure to the cytokine stimuli, such as IL-12, IL-15 and IL-18, MAIT cells demonstrate a remarkable ability to generate elevated levels of IFN- γ (Hinks & Zhang, 2020). This activation mechanism enables MAIT cells to react to microorganisms that do not generate MR1 ligands, such as viruses or bacterial strains that do not synthesize riboflavin (van Wilgenburg et al., 2016; Ussher et al., 2014). Such TCR-independent activation characteristic is also a common trait observed in other innate-like T cell subsets, including $\gamma\delta$ and iNKT cells (Hinks & Zhang, 2020). In the presence of the antigen, a synergistic interplay between TCR and cytokine signals triggers the activation of MAIT cells, which propels MAIT cells to execute their cytolytic functions,

employing the secretion of granzymes and perforin to initiate cell lysis and apoptosis (Figure 1.4). Besides, MAIT cells engage in the pro-inflammatory response by releasing a spectrum of cytokines, including IL-2, IL-17, IFN- γ , and TNF- α , collectively enhancing the immune responses (Figure 1.4) (Vacchini et al., 2020).

To comprehensively explore the attributes and functional behaviours of MAIT cells, current research employs a combination of TCR-dependent and TCR-independent methods for their activation and *in vitro* culture. The TCR-dependent methods include activation through anti-CD3/anti-CD28 antibodies and corresponding cytokines, and co-culture assays using irradiated PBMCs or microbial-fed feeder cells, such as paraformaldehyde (PFA)-fixed *E. coli* loaded on THP-1 cells (Hagel et al., 2020; Parrot et al., 2021). The TCR-independent activation methods involve direct stimulation via recombinant cytokines, such as IL-12 and IL-18, as well as indirect activation using Toll-like receptor 4 (TLR-4) or Toll-like receptor 8 (TLR-8) agonists, or through exposure to influenza A virus in the presence of APCs (Hagel et al., 2020). Depending on the research interest and experimental inquiries, a spectrum of activation strategies has been selected to stimulate MAIT cells *in vitro*.

1.3.3. The role of MAIT cells in infection and tissue repair

As a distinct subset of innate-like T cells, MAIT cells demonstrate an exceptional responsiveness to a diverse range of bacteria and microorganisms, depending on the presence of the riboflavin-biosynthesis pathway within these entities. This unique property positions them as key components of host defence against infections within peripheral tissues (Hinks & Zhang, 2020). Upon pathogen stimulation, MAIT cells are known to produce cytokines such as IFN- γ , TNF- α , granzyme B, perforin, IL-2, and IL-17, while typically not secreting IL-10. Human MAIT cells display a mixed Th1/Th17 cytokine profile, which can vary depending on their location within the periphery, reflecting potential local environmental influences (Hagel et al., 2020; Howson, Salio & Cerundolo, 2015). Studies using MR1 knockout mouse models have revealed the critical functional role of MAIT cells during infection. Mr1^{-/-} mice lack MAIT cells due to the requirement of MR1 recognition for their thymic development. For instance, in a mouse model of sepsis, MAIT cell-deficient (MR1^{-/-}) mice exhibited significantly higher mortality associated with an increased burden of bacteria in the blood compared to wild-type mice (Trivedi et al., 2020). Similarly, in response to severe H1N1 influenza, MR1^{-/-} mice showed enhanced weight loss and mortality, whereas adoptive transfer of MAIT cells led to improved disease outcomes in the immunocompetent mice model, highlighting the protective capacity of MAIT cells in respiratory viral infections and suggesting their potential as therapeutic targets (Wilgenburg et al., 2018).

Beyond their role in infection to prevent any immune threats, MAIT cells also contribute to the maintenance of tissue homeostasis, promotion of tissue repair and wound healing. MAIT cells are distributed throughout various tissues, where they can influence local immune responses and tissue repair processes. In the gut, IL-17 plays a role in regulating Occludin to

maintain appropriate barrier function and prevent excessive permeability during epithelial injury (Nel et al., 2021). Besides, MAIT cells in the meninges express abundant antioxidant molecules. When MAIT cells are deficient in mice, this leads to an increase in reactive oxidative species in the meninges, resulting in meningeal barrier leakage. Adoptive transfer of MAIT cells effectively prevented meningeal leakage in $Mr1^{-/-}$ mice, suggesting their role in preserving meningeal barrier homeostasis and integrity (Zhang et al., 2022). Recent findings have also elucidated the role of MAIT cells in wound healing. During the repair phase of skin injury, circulating MAIT cells migrated to the wound site in a CXCR6-dependent manner. At the wound site, MAIT cells contributed to wound repair by secreting amphiregulin and promoting the proliferation of keratinocytes. This process accelerated the closure and repair of the skin following injury, underscoring the importance of MAIT cells in tissue regeneration and wound healing (du Halgouet et al., 2023). Additionally, experiments using $Tcrd^{-/-}$ $Mr1^{-/-}$ mice lacking MAIT cells revealed delayed healing of skin punch biopsies, which was subsequently improved by topically applying the MAIT cell ligand, 5-OP-RU, before the injury (Constantinides et al., 2019).

The multifaceted roles of MAIT cells are being increasingly characterized, showcasing their versatility and importance in maintaining overall health. On one hand, MAIT cells promptly respond to bacterial and microbial challenges, exemplifying their critical role in host defence. Through recognition of riboflavin metabolites, MAIT cells produce cytokines and exert cytotoxic effects, contributing to pathogen clearance and immune regulation. On the other hand, MAIT cells also play pivotal roles in tissue repair and homeostasis. Their ability to migrate to injured sites, secrete factors like amphiregulin, and promote keratinocyte proliferation underscores their significance in wound healing and tissue integrity.

Understanding the delicate roles of MAIT cells offers promising insights for therapeutic interventions aimed at modulating MAIT cell responses for improved health outcomes. Further exploration into the mechanisms governing MAIT cell behaviour will undoubtedly unveil new dimensions of their roles in health and disease.

1.3.4. The role of MAIT cells in cancer

Extensive studies have explored the multifaceted roles of MAIT cells across a spectrum of conditions, including microbial infections, inflammation, and autoimmune disorders. With the tide of scientific interest shifting, a novel spotlight now focuses on the unexplored role of MAIT cells in the field of malignancy and cancer.

Various investigations propose a potential involvement of MAIT cells in tumour immunity. Initial report indicating MAIT cell relevance in cancer revealed that the gene encoding V α 7.2 (TRAV1-2) and J α 33 (TRAJ33) transcripts are enriched in brain and kidney tumours and consistently present in tumour-infiltrating lymphocytes (Peterfalvi et al., 2008). In a meta-analysis of exploring the genes and infiltrating immune cells across various human cancers, the presence of KLRB1 (CD161 encoding gene) in leukocytes infiltrating tumours emerged as the most favourable prognostic indicator and marker of enhanced innate immune features across 30 different cancer types (Gentles et al., 2015). While the expression of CD161 is shared by activated cytotoxic CD8 T cells and NK cells, it's noteworthy that MAIT cells constitute a significant proportion of CD161⁺ T cells infiltrating peripheral regions (Ussher, Klenerman & Willberg, 2014). These findings suggest a promising prospect wherein MAIT cells can infiltrate into the tumour sites and are involved in tumour immunity. However, the precise underlying molecular mechanisms have yet to be elucidated.

Subsequent studies have confirmed the abundance of MAIT cells in certain tumour microenvironment and demonstrated their natural preferential migration toward some cancer niches, including melanoma, gastric carcinoma, colorectal carcinoma, and pulmonary neoplasms (Li et al., 2023; Park & Kee, 2015; Vacchini et al., 2020). This aligns with the report

of a relatively diminished frequency of circulating CD8⁺ MAIT cells in individuals with solid tumour in comparison to the healthy control cohort, suggesting the plausible scenario of CD8⁺ MAIT cell migration from the periphery towards tumour sites (Ling et al., 2016). However, the present clinical data concerning the influence of MAIT cells on cancer prognosis is contradictory, given that both negative and positive effects of MAIT cells have been witnessed in both solid tumours and hematologic malignancies. In melanoma, patients with high frequencies of CD8⁺ MAIT cells exhibited better respond to immune checkpoint inhibitor therapy and significantly improved overall survival, while the non-responding patients remain low in MAIT cell count (Vorwald et al., 2022). Distinct studies conducted on MAIT cells extracted from the peripheral blood of healthy individuals have also demonstrated their capability for direct cytotoxicity towards malignant cells following activation, including oesophageal cancer, multiple myeloma, and colorectal cancer (Gherardin et al., 2018b; Melo et al., 2019; Ling et al., 2016). The cytotoxicity of MAIT cells against cancer cells occurs primarily via exocytosis of cytotoxic granules containing perforin and granzyme in an either MR1-dependent or MR1-independent manner (Won et al., 2016). In mouse model, MR1-restricted T cells can also kill leukaemia cells *in vivo* by identifying of the cancer metabolome and prolong the survival of mice (Crowther et al., 2020). Therefore, it has been speculated that MAIT cells may be the new killer cells in anti-tumour immunity.

However, in colorectal cancer and hepatocellular carcinoma, there is a negative association between high frequencies of infiltrating MAIT cells and positive clinical outcomes (Zabijak et al., 2015; Shaler et al., 2017). Unlike the healthy liver tissue MAIT cells, these penetrating MAIT cells exhibit wide-ranging dysfunctions, including their impaired ability to produce IFN- γ and granzyme B but higher levels of IL-17. IFN- γ possesses the ability to modulate the p53

signaling pathway, prompting cell cycle arrest progression and apoptosis in tumour cells, while overall IL-17 promotes pro-tumour inflammation, angiogenesis, and metastasis in individuals with early stages of colorectal cancer (Zabijak et al., 2015). Research findings have also demonstrated the presence of MAIT cells in colon tumours with a terminally exhausted phenotype characterized by elevated levels of PD-1, TIM-3, and CD39. These exhausted MAIT cells exhibit diminished polyfunctionality, leading to a decrease in the production of key effector molecules essential for effective anti-tumour responses (Rodin et al., 2021). Therefore, the tumour microenvironment has the potential to trigger exhaustion and dysfunction not only in conventional T cells, but also in MAIT cells. Consequently, the compromised functionality of MAIT cells within the tumour milieu could result in suboptimal regulation of cancer immunity and, in the most severe scenario, contribute to cancer progression. Blockade of the PD-1 receptor can restore MAIT cell frequency and function in patients to enhance anti-tumour effects and overall survival, indicating the potential of MAIT cells as immunotherapeutic target in cancer (Melo et al., 2019; Rodin et al., 2021). Additionally, when discussing MAIT cell roles in cancer, it's essential to note that previous reports haven't consistently categorized MAIT cells into subgroups based on their CD4 and CD8 expression. This lack of subgroup categorization may contribute to discrepancies regarding the functions of MAIT cells in the disease. From the clinical reports, only the circulating CD8⁺ MAIT cells but not CD4⁺ are associated with favourable clinical results, implying great distinctions in functions between the MAIT cell subsets (Vorwald et al., 2022). CD4⁺ MAIT cells demonstrated increased level of FOXP3 expression in the resting state relative to the CD8⁺ subset. The co-expression of FOXP3 and CD25 on the surface of CD4⁺ MAIT cells resembles that of CD4⁺ Tregs, raising the possible speculation that CD4⁺ MAIT cells

might possess similar immune regulatory properties, potentially contributing to the adverse clinical outcomes observed in cancer (Vorkas et al., 2022).

Taken together, these results highlight the intricate modulation of MAIT cell responses in cancer. MAIT cells exhibit a remarkable capacity to infiltrate tumour sites, recognize tumour-associated antigens, and trigger targeted immune responses, suggesting their pivotal role in cancer immunosurveillance. However, their multifaceted nature in cancer, with the potential to be both pro- and anti-tumorigenic under varying circumstances, poses a challenge. Further investigation into the functional characteristics of each MAIT cell subset is warranted to elucidate their precise role in immune modulation and their impact on disease progression and treatment outcomes. Ongoing endeavours in research and clinical investigations are essential to fully harness the therapeutic potential of MAIT cells in the battle against cancer.

1.4. B cell malignancies

B cell malignancies represent a set of B cell-associated hematologic disorders, including B-cell lymphomas and leukaemia, characterized by abnormal growth and uncontrolled B cell proliferation, resulting in the disruption of normal immune function. Non-Hodgkin B cell lymphoma (NHL) is one of the most prevalent haematological malignancies worldwide, representing approximately 3% of cancer diagnose and mortality (Thandra et al., 2021). It comprises a diverse spectrum of disorders, including indolent and aggressive subtypes. The indolent ones could be kept in remission after treatment with long-term survival. Aggressive lymphomas are defined as a disease that tends to grow and spread rapidly, causing severe symptoms. The common types of B cell malignancies include diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL), chronic lymphocytic leukaemia (CLL), and marginal-zone lymphomas (MZL) (Salles et al., 2017). The exact causative factors contributing to the development of B cell malignancies are intricate and not fully understood. Nevertheless, numerous elements have been linked to an elevated susceptibility to these conditions. These factors encompass genetic alternations, exposure to radiation, virus-associated hits, such as Epstein-Barr virus and hepatitis C, that eventually lead to cancerous transformation in B cells. Persistent B cell stimulation resulting from autoimmune disorders or certain infections can also potentially facilitate the progression toward malignancy (Seifert, Scholtysik & Küppers, 2019; Thandra et al., 2021).

1.4.1. Current treatment landscapes

Current treatment approaches for B-cell malignancies encompass a spectrum of strategies, including chemotherapy, radiation therapy, targeted therapies, and stem cell transplantation. Standard initial treatments for B cell lymphomas frequently involve CHOP (combination of cyclophosphamide, doxorubicin, vincristine, and prednisone) and other chemotherapy regimens, leading to curative outcomes in approximately 50–70% of patients. However, a substantial proportion of individuals may encounter relapse or refractory disease (Miao et al., 2019). Allogeneic hematopoietic stem cell transplantation (HSCT) holds a potential as a therapeutic option, yet its applicability is constrained by factors such as advanced age, comorbidities, and HSCT-related mortality (Kocher et al., 2020). The advent of antibody-based therapies and small molecule inhibitors has improved the clinical outcomes of patients with advanced B cell malignancies in the last decade. In particular, the antibody rituximab targeting the B cell transmembrane protein CD20 has a dramatic effect on elimination of malignant B cells through antibody-dependent cellular cytotoxicity and FCγR-mediated phagocytosis (Salles et al., 2017). The development of small molecule, such as Bruton's tyrosine kinase (BTK) inhibitor ibrutinib and PI3K inhibitor, also contributes to the management of cancer via disrupting the signalling pathway of lymphoma cells (Ayyappan & Maddocks, 2019). Despite favourable responses in some patients, these treatments can be accompanied by toxicity, adverse effects and may not offer enduring remission for many, emphasizing the imperative need for more efficacious and precisely targeted therapies.

1.4.2. Advances of CAR-T cell in treating B cell lymphoma

In the last decade, the range of treatment choices available to patients diagnosed with B-cell lymphomas has significantly broadened due to the approval of various novel treatment classes. Development of CAR-T cell therapy now becomes the one of most significant advances in cancer immunotherapy. CAR-T cells, engineered to target specific B cell antigens, particularly CD19, have demonstrated remarkable efficacy in inducing durable remissions in patients with B cell lymphoma. After CAR-T cell infusion, the overall survival of patients with aggressive B cell lymphoblastic leukaemia at 6 months was over 78% and the rate of event-free survival was 50% at 12 months. Remarkably, long-term remission of up to 2 years could be achieved due to the CAR T-cell persistence in these patients (Maude et al., 2018). The improved survival and safety of the CAR-T cell therapy has also been reported by the other parallel clinical studies, with an overall remission rate of 89% observed in patients with r/r CD19⁺ ALL, and a relatively low incidence of severe CRS, affecting only 23% of the participants (Gardner et al., 2017). In response to the potential relapse and escape of CD19 on malignant cells, researchers have also begun exploring CD20 CAR-T cells as an alternative or complementary therapy (Shadman et al., 2020; Shah et al., 2020). As another B cell surface marker expressed on the majority of B cells, targeting CD20 can provide an additional strategy to overcome resistance or cancer relapse observed with anti-CD19 CAR-T cell therapy (Shadman et al., 2020). The development of CD19/CD20 bispecific CARs has been undertaken to address this challenge, aiming to enhance the efficacy and broaden the applicability of CAR-T cell therapy in B cell malignancies. Clinical studies have reported that dual-targeting of CD19 and CD20 using the bispecific CARs is safe and effective in patients with relapsed/refractory lymphomas (Ghafouri et al., 2020; Larson et al., 2023).

As mentioned in the previous part, FDA approval of the commercial CAR T-cell products against B cell malignancies has validated their effectiveness and allowed for broad access to CAR-T cell therapy. The remarkable achievements of CD19-specific CAR-T cells in B cell malignancies serve as a compelling catalyst for continued exploration and advancement of CAR-based therapies for cancer. Given the well-established foundation of CAR immunotherapy exemplified by CAR-T cells, their development, successful clinical translation, and demonstrated effectiveness in B cell malignancies offer a valuable roadmap for investigating and developing the novel CAR-MAIT cell therapy.

1.5. Hypotheses

MAIT cells obtained from healthy donors can be expanded *in vitro* and genetically modified with CAR to target specific tumour antigens. The CAR-modified MAIT cells could demonstrate comparable levels of cytotoxicity and effectiveness as the conventional CAR-T cells when targeting the specific cells. As MAIT cells pose a low potential of alloreactivity, they could serve as a novel therapeutic approach for the treatment of B cell malignancy in allogeneic settings.

1.6. Aims and objectives

The overall aim of this project is to explore the potential of MAIT cells as a novel source for CAR-T cell therapy and to evaluate their efficacy in the preclinical settings. This study is guided by three specific sub-aims:

1. Develop an effective CAR construct designed to target malignant cells, such as B cells, and establish an efficient transduction protocol for delivering the CAR gene into MAIT cells.
2. Evaluate the killing capacity of CAR-MAIT cells against target cells and investigate their suitability for allogeneic therapy by assessing their reactivity to allogeneic stimulation.
3. Develop novel approaches to specifically expand and scale up MAIT cell production, aiming to overcome limitations related to their low frequency in PBMCs.

By addressing these sub-aims, this project seeks to advance our understanding of MAIT cell biology and their potential applications in CAR-T cell therapy. The findings from this study may contribute to the development of innovative immunotherapeutic strategies for the treatment of cancer and other diseases.

Chapter 2. Materials and Methods

2.1. Cell culture

2.1.1. Maintenance of cell lines

Table 2.1 shows the cell lines used in this thesis. The culture medium for the adherent cells was composed of 90% high glucose Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine (Gibco, UK) (D10). The adherent cell lines were passaged once the cell confluence reaches 80 to 90%. For general cell maintenance, cells were split every 2-3 days. Trypsin-ethylenediaminetetraacetic acid (0.25% Trypsin-EDTA, Gibco) was used to dissociate the adherent cells from the flasks or plates. After incubation for 2 to 5 minutes, 2 volumes of D10 were added to neutralize the trypsinization. The cells were collected and centrifuged at 500 g for 5 minutes. The cell lines were passaged based on their replication rate. Cell lines like Plat-GP and 293T were passaged at a ratio of 1:5. PG-13 were passaged at a ratio of 1:10 due to their faster growth rate.

For the suspension cell lines, such as B cell line, they were cultured in the R10 medium, containing 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine and 90% Roswell Park Memorial Institute Medium 1640 (RPMI 1640) (Gibco). The BLCLs were generated by infecting B lymphocytes with EBV, leading to their transformation into immortalized EBV-bearing BLCLs. The BLCLs utilized in this study were generously provided by the previous PhD student of our lab, Alessia Dalla Pria. To maintain these cells, a regular splitting schedule was adopted, involving the removal of four-fifths of the total cell suspension every 3 days, followed by supplementation with fresh R10 medium.

The cell number and viability were measured using Vi-CELL XR Cell Viability Analyzer (Beckman Coulter, UK). 60 μ L of cell suspension was added into 540 μ L PBS as a 10-fold dilution for analysis.

Table 2.1. Cell lines used in this thesis.

Cell line	Description	Source
Platinum-GP (Plat-GP)	Transient retroviral packing cell line	Cell Biolabs
PG13	Stable retroviral packaging cell line	ATCC
293T	Epithelial-like cell line	ATCC
BLCL	B cell lymphoblastoid cell line	CIV lab

2.1.2. Cryopreservation and revival of cells

For long-term preservation, cells were stored in liquid nitrogen. The cells for cryopreservation were collected and centrifuged at 300 g for 5 minutes. The cell pellets were resuspended in the freezing medium containing 90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO). Then the cell suspension was aliquoted into the sterile Cryotubes (Corning, United States) and placed into a 4°C prechilled an isopropanol-filled freezing container or Mr. Frosty (Thermo Fisher Scientific, UK). The tubes were then stored in a -80°C refrigerator overnight. On the following day, the vials were ready to be transferred to liquid nitrogen for long-term storage.

To thaw and recover cells from cryopreservation, cells were taken out from liquid nitrogen and transferred by dry ice. The cryotubes were put immediately into the 37°C water bath. Agitation was carried out in a swirling fashion for an even distribution of heat. To remove

DMSO in the cryopreservation solution, cells were added dropwise into the warm 37°C medium with FBS (Sigma-Aldrich, UK). The medium was 10 volumes to the amount of the thawed cell suspension added. Cells were washed with the medium and centrifuged at 500 g for 5 minutes to discard the supernatant. Following resuspension of cells in the desired amount of medium, cells are ready for the next step application.

2.1.3. Blood processing for PBMCs isolation

The blood from the healthy donors was acquired from the NHS transfusion unit in the form of leukocyte cones, which are produced as the by-products of the apheresis process for research use. The leukocyte cones contain a high density of leukocytes concentrated in a volume of 10-15 mL. PBMCs were isolated from blood via density gradient centrifugation over Histopaque (Sigma-Aldrich). 15 mL Histopaque was added into the 50 mL SepMate™-50 (Stemcell Technologies, UK) through the central hole of the insert. The top of the Histopaque would be above the insert. The blood was diluted by 1 to 2 volumes of PBS with 2% FBS and added into the SepMate™ by pipetting it down the side of the tube. The diluted blood would mix with the density gradient medium above the insert and the tubes were centrifuged at 1200 g for 10 minutes at room temperature with the brake on. By using the SepMate™ tubes, the centrifugation time could be largely reduced and the interface between the blood and Histopaque is less likely to be disturbed due to the design of the insert. The top layers containing enriched mononuclear cells (MNCs), comprising lymphocytes and monocytes, could be poured off into a sterile new tube. To remove the remaining Histopaque, at least two washes with PBS + 2% FBS were performed after 300 g centrifugation for 8 minutes at room temperature and discard the supernatant. The isolated PBMCs are then ready for use or cryopreservation.

2.1.4. T cell activation and expansion

Conventional T cells from isolated human PBMCs were activated by Dynabeads™ Human T-Activator CD3/CD28 (Thermo Fisher Scientific) at a 1:1 bead-to-cell ratio. CD3/CD28 Dynabeads were washed by FBS before adding into PBMCs. T cells were plated at a density of 1×10^6 /mL in the culture. For example, 1×10^6 T cells were cultured in 1 mL of R10 medium in one well of a 24-well sterile cell culture plate. Cytokines, such as 16 ng/mL human IL-2 (Miltenyi Biotech, UK), 10 ng/mL human IL-7 (Miltenyi Biotech) and IL-15 (Miltenyi Biotech), were supplemented and replenished every 2 to 3 days. T cells can be expanded for circa 14 to 15 days before harvest.

2.1.5. MAIT cell isolation

In the purified MAIT cell assay, naïve MAIT cells were isolated from unstimulated PBMCs using Magnetic cell sorting (MACS). MR1 tetramers were used to specifically label MAIT cells, as the tetramers contain a fluorescently labelled MR1 molecule loaded with the MAIT cell-specific antigen, such as 5-OP-RU. Briefly, 5 µg aliquot of biotinylated 5-OP-RU-loaded MR1 tetramers (kindly provided by Prof. James McCluskey, University of Melbourne, Australia) were added to a total volume of 18 µL in PBS buffer. To make PE-conjugated MR1 tetramer solution, commercially available streptavidin-PE at 0.5 mg/mL (Biolegend, UK) should be combined with Tris-buffered saline to obtain a total volume of 17 µL. 1/10 of the streptavidin-PE solution (1.7 µL) should be added to the MR1 solution every 10 minutes and gently mixed, while the solution is kept at room temperature in the dark between each step. This step should be repeated until all streptavidin-PE solution has been added. This process yielded a final volume of 35 µL containing 0.143 µg/µL of tetramer, which should be titrated before use, with

dilutions ranging from 1:200 to 1:1000. The solution could be stored at 4°C for days to weeks. The original MR1 tetramer aliquots without PE labelling should be stored at -80°C until use. To isolate MAIT cells, PBMCs were labelled with PE-conjugated 5-OP-RU-loaded MR1 tetramer for 30 minutes and washed with PBS + 5% FBS to remove the unbound tetramers. Anti-PE Microbeads (Miltenyi Biotech) were mixed with the cell samples to bind to the labelled cells. The cell suspension was resuspended in 80 µL of buffer per 10^7 total cells, and 20 µL of corresponding microbeads per 10^7 total cells were added. The cell mixture was subjected to separation in a MACS column placed in a MACS separator (Miltenyi Biotech), following the manufacturer's instructions. For cell numbers within 2×10^8 cells, a positive selection MS column (Miltenyi Biotech) was used for separation, while for cell numbers over 2×10^9 cells, a positive selection LS column (Miltenyi Biotech) was used. Prior to applying the cell suspension, the MS column was rinsed with 500 µL buffer (3 mL for LS column). The unlabelled cells that passed through the column were collected, and the total effluent was the unlabelled cell fraction. The column was washed with the appropriate amount of buffer for three times, adding new buffer only when the column reservoir was empty. The purified and isolated MAIT cells are ready for use or cryopreserved.

2.1.6. Antibody coating and stimulation

In the context of PBMC assay using antibodies, 5 µg/mL of anti-Vα7.2 antibodies, including clones REA179 (Biolegend), 3C10 (Beckman Coulter), and OF5A12 (BD Biosciences, UK), or anti-CD3 antibody clone OKT3 (Thermo Fisher Scientific), with/without the functional grade anti-CD28 antibody clone CD28.2 (Thermo Fisher Scientific) were coated onto the 96-well cell polypropylene culture plates (Corning). The antibodies were diluted in PBS to achieve the desired concentration. 100 µL of the diluted antibodies were added to the plate and incubated overnight at 4°C. Following the incubation, any unbound antibodies were removed by washing the plates 2-3 times with PBS. On the day of stimulation, 2×10^6 cells/mL were added to the plates in the presence of 16 ng/mL human IL-2 (Miltenyi Biotech, UK).

2.1.7. Generation of 5-OP-RU loaded MR1-tetramer beads

In the context of PBMC assay, 5-OP-RU loaded MR1-tetramer beads (referred as 'MR1 beads') were generated in our lab to access the stimulation effects on MAIT cells. The MR1 beads were used as artificial APCs to present the MAIT-specific antigen 5-OP-RU to MAIT cells in a MR1 tetramer dependent manner. The synthesized MR1 ligand, 5-OP-RU, and the biotinylated 5-OP-RU loaded MR1 molecules were kindly provided by Prof James McCluskey, University of Melbourne, Australia. In brief, the MR1 beads were generated by immobilizing the biotinylated 5-OP-RU loaded MR1 molecule and biotin-conjugated anti-human CD28 (CD28.2) antibody (Thermo Fisher Scientific) onto the 2.8 µm cell-size magnetic Dynabeads M-280 Streptavidin (Thermo Fisher Scientific) at different ratios, as shown in Table 2.2. In brief, 3×10^7 streptavidin beads were rinsed in PBS. Different ratios of anti-human CD28 antibody and MR1 molecules were mixed to 1 mL PBS without Ca^{2+} or Mg^{2+} (Sigma-Aldrich).

The vial containing the beads was placed on a magnet to remove the washing buffer. The mixture of antibody/protein was immediately added to the beads and inverted to mix. The vial was then covered with parafilm and incubated overnight at 4°C on a rotator. Excess unbound protein was removed by washing with PBS. The MR1 tetramer/anti-CD28 conjugated beads can be stored in PBS at 4°C for weeks. The conjugation of MR1 tetramers and anti-CD28 to the beads was confirmed by staining the beads with anti-human MR1 antibody (Biolegend) and goat anti-mouse IgG (Thermo Fisher Scientific) and analysing with flow cytometry. The prepared MR1 beads were added to PBMCs at 1:1 bead-to-cell ratio in the presence of various cytokines, including 16 ng/mL IL-2, 10 ng/mL IL-7, 20 ng/mL IL-12, 10 ng/mL IL-15, 20 ng/mL IL-18 and 20 ng/mL IL-23 (Miltenyi Biotech), depending on the experiments.

Table 2.2. Production of MR1-loaded artificial APC beads at different CD28:MR1 tetramer ratios (total 5 µg/3x10⁷ beads)

CD28:MR1 tetramer ratio	Anti-human CD28 antibody (µg)	5-OP-RU loaded MR1 tetramer (µg)
0:4	0	5
1:3	1.25	3.75
2:2	2.5	2.5
3:1	3.75	1.25
4:0	5	0

2.2. Molecular biology techniques

2.2.1. Cloning of CD19-specific CAR genes into the retrovirus vector

The schematic graph of CD19-specific CAR used in this thesis is displayed by Figure 2.1. The DNA sequence of the mouse IgG antibodies FMC63 (anti-CD19) was obtained from the public sources (US6410319B1). DNA of FMC63 single-chain variable fragments (scFvs) was synthesised by Genscript, UK and then fused with a CD8 transmembrane domain, a CD28 and a 4-1BB endodomain as co-stimulatory signal, and the CD3 ζ chain for activation. To facilitate CAR detection, the gene sequence of tEGFR was tagged behind CAR via self-cleaving 2A linkers. In brief, the restriction enzymes *NcoI* and *NotI* (New England Biolabs, UK) were used to linearize the template MSCV vector established by our lab (Ma, 2019). The synthesized scFv fragment of FMC63 was cleaved by the same restriction enzymes *NcoI* and *NotI* to generate compatible overhangs for binding. The conditions of double restriction enzyme digest reaction are outlined in Table 2.3. The reaction was performed either at room temperature overnight or at 37°C for 1-2 hours. After the reaction, the linearized vector and the cleaved inserts were separated by electrophoresis on a 1% agarose gel at 100V for 1 hour to purify the desired fragment. The purified DNA fragments were then eluted from the gel using QIAquick Gel Extraction Kit (Qiagen, UK), following the manufacturer's instruction. The extracted DNA was quantified by the NanoDrop Spectrophotometer (Thermo Fisher Scientific).

The T4 ligase (New England Biolabs) was utilized to perform the ligation reaction between each insert and vector backbone. The reaction conditions are outlined in Table 2.4. The T4 ligase buffer and DNA to be ligated were thawed on ice. The DNA fragments were then mixed

in the desired molar ratios in a sterile microcentrifuge tube. The amounts of DNA required for ligation at a molar ratio (insert: vector = 3:1) was calculated on the New England Biolabs website, depending on the size of the target vector and inserts. The reaction mixture was incubated at 16°C to 22°C overnight to allow ligation to occur.



Figure 2.1. Cloning of the 3rd generation CAR construct.

The schematic diagram of CAR construct. The scFv derived from monoclonal antibody FMC63 was fused with a CD8 transmembrane domain (TM) followed by CD28 endodomain, 4-1BB and CD3 ζ intracellular domains. EGFR was tagged behind CAR via self-cleaving 2A linkers. SP: Igk chain leader sequence; scFv: single-chain variable fragment; TM: transmembrane motif.

Table 2.3. Double restriction enzyme digest reaction of MSCV vector and scFv fragments.

Reagent	Volume
Plasmid DNA	10 μ g
CutSmart Buffer 10X	10 μ L
NcoI (HF)	2.5 μ L
NotI (HF)	2.5 μ L
Nuclease-free water	To 100 μ L final volume

Table 2.4. T4 ligation reaction between the insert and vector backbone.

Reagent	Volume
T4 buffer	2 μ L
Vector DNA (8kb)	50 ng
Insert DNA (700 bp)	14.65 ng
T4 ligase	1 μ L
Nuclease-free water	To 20 μ L final volume

2.2.2. Cloning of CD19-specific CAR genes into the lentivirus vector

To clone the CD19-targeting CAR genes into the lentiviral vector, a PCR-based approach was employed to obtain the CAR construct sequences from the retroviral vector. Subsequently, these PCR-amplified sequences were integrated into the linearized lentiviral vector using infusion cloning. The process involved a two-step PCR amplification utilizing Phusion polymerase (New England Biolabs) and was carried out on a ProFlex PCR system (Thermo Fisher Scientific). The specific PCR conditions are detailed in Table 2.5 and Table 2.6. The primer sequences employed are provided below:

Forward primer: 5'-GCCCCGGGGCCGCCACACCATGGAGACAGACACACTCC-3'

Reverse primer: 5'-TCAAGCTTGCATGCCTCACATGAAGAGGCCGATCCC-3'

Table 2.5. The components of PCR.

Reagent	Volume
Plasmid DNA	10 ng
GC buffer	10 µL
10 nM dNTPs	1 µL
10 µM Forward primer	2.5 µL
10 µM Reverse primer	2.5 µL
Phusion polymerase	0.75 µL
Nuclease-free water	To 50 µL final volume

Table 2.6. The conditions for two-step PCR.

Step	Temperature	Time
Initial denaturation	98°C	30 seconds
30 cycles	98°C	10 seconds
	72°C	30 seconds per kb
Final extension	72°C	10 minutes
Hold	4°C	

The lentivirus template vector LA-05-2 was kindly provided by Dr Weiwei Ma from Unicet Biotech company, China. Restriction enzymes *SbfI* and *BstXI* (New England Biolabs) were used to linearize the vector at 37°C for 1-2 hours. The linearized vector and the PCR products were separated by electrophoresis on a 1% agarose gel and eluted from the gel using QIAquick Gel Extraction Kit (Qiagen). In-Fusion Snap Assembly kit (Takara Bio, UK) was used to ligate the linearized vector and the purified PCR products, shown by Table 2.7. The molar ratio of the vector to insert was determined using Takara's online cloning tools, which considered the respective base pair numbers of each component. The In-Fusion reaction was incubated at 50°C for 15 minutes and then placed on ice. The cloning reaction products could be stored at -20°C until *E.coli* transformation.

Table 2.7. The components of In-Fusion Snap Assembly cloning reaction.

Reagent	Volume
5X In-Fusion Snap Assembly Master Mix	2 μ L
Linearized vector (11373 bp)	133 ng
Purified PCR fragment (2878 bp)	67 ng
Nuclease-free water	To 10 μ L final volume

2.2.3. Transformation of stable competent *E.coli*

The products of ligation mixture was transformed into the competent bacteria, *E.coli* DH5a (Invitrogen, UK). To initiate the transformation, 30 μ L of the competent bacteria was thawed on ice and 3 μ L of the ligation products was added, gently mixed, and placed on ice for 30 minutes before being heat shocked at 42°C for 30 seconds. After the heat shock, the *E.coli* was incubated on ice for 2 minutes and then mixed with 570 μ L of Super Optimal Broth (SOC) medium (Thermo Fisher Scientific) at room temperature. The cells were then incubated at 37°C for 1 hour with shaking at 250 rpm. Following a 1-hour recovery period, 25 to 50 μ L of the *E.coli* mixture was spread on the ampicillin LB plates and incubated at 37°C for overnight. The LB agar plates were made from the LB agar plate powder (Sigma-Aldrich) and 1:1000 (v/v) ampicillin solution (Sigma-Aldrich).

2.2.4. Extraction and screening of the correct plasmids

On the following day of *E.coli* transformation, a single clone was selected and grown in 5 mL of LB medium. The plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen), following the manufacturer's protocol. The extracted recombinant DNA was verified through double restriction digest analysis by *NcoI* and *NotI* (New England Biolabs). After gel electrophoresis, the sample produced two correct bands (indicating the 700 bp insert fragment and 8kb to 10kb vector) from the miniprep plasmid digests were prepared for DNA sequencing by Genewiz, UK. To obtain large amounts of purified vector for subsequent research use and transfection, the bacterial clones carrying the accurate DNA were expanded in 100 mL of LB medium overnight at 37°C and the plasmids were extracted by the QIAprep Spin Maxiprep Kit (Qiagen). The concentration of extracted DNA was quantified using the NanoDrop Spectrophotometer (Thermo Fisher Scientific). The *E.coli* clones were stored at -80°C in 20% autoclaved glycerol.

2.2.5. DNA synthesis and sequencing

SnapGene software was used to design the CAR inserts and vectors. The scFv sequences of FMC63 with two desired restriction sites were sent to Genscript for custom-made synthesis. The extracted DNA samples from the miniprep were sent to Genewiz for sequencing. The results were analysed and aligned by SnapGene version 4.3.7.

2.3. CAR transduction

2.3.1. Production of CAR-expressing retrovirus

To achieve high transduction efficiency to primary human T cells, a two-step retroviral system involving the sequential use of PlatGP and PG13 cell line was developed. The Plat-GP cell line (Cell Biolabs, UK) is a type of retroviral packaging cell line used to produce retroviral particles. It was derived from the 293T cell line and modified with the addition of *gag* and *pol* genes to enhance retroviral yield. The Plat-GP cell line was transfected with retroviral vectors by co-transfection with VSV-G envelope vector (Addgene, UK) and vector containing the desired CAR-expressing genes to produce retroviral particles, and the produced particles were used to infect another cell line for a stable virus-producing cell line. The PlatGP cell lines were recovered from cryopreservation and cultured in D10 medium for 5 days before transfection. Cells were passaged at a ratio of 1:5 when they reached 90% confluence by trypsinization. On the day before transfection (Day 0), 1.2 million PlatGP cells were plated in one well of six-well plate with 2 mL D10 medium. On the day of transfection, the wells containing cells with 80-90% confluence would be used for transfection. To make the transfection solution, 200 µL of Opti-MEM (Gibco), a serum-free medium, was added to the tubes for each transfection reaction. Then, the vectors were added, including 0.5 µg of VSV-G vector and 2 µg of MSCV-FMC63-EGFRt vector. After a 5-minute incubation at room temperature, 7 µL of X-tremeGENE HP Transfection Reagent (Sigma-Aldrich) was added, and the solution was incubated at room temperature for 15 minutes. The solution was then added dropwise to the target well for transfection, and the cells were returned to the 37°C cell incubator. After 18 hours, the medium was replaced with 2 mL fresh D10. Retrovirus particles were produced and could be harvested from the supernatant after 48 hours and 72 hours post-transfection. To harvest the

viral supernatant, the media was collected and centrifuged at 300 g for 5 minutes to exclude floating cells.

Since PlatGP cells can only survive for 3-4 days after transfection, they were not used as a stable source of retroviral vector production. Instead, a stable transfection retroviral packaging cell line, PG13 (ATCC, UK), was used for this purpose. Similar as the Plat-GP cell line, the PG13 cells were cultured in D10 medium for 5 days before transduction. The cells were passaged at a 1:10 ratio when they reached 90% confluence by trypsinization. To prepare for transduction, 0.3 million PG13 cells were plated in 6-well plate wells the day before to achieve 30% confluence. On the day of transduction, viral supernatant from PlatGP cells was thawed on ice, and polybrene (Sigma-Aldrich) was added to the virus at a concentration of 6 µg/mL. Polybrene can neutralize the charge on the surface of the virus and target cell surface, enhancing their contact and increasing the efficiency of transduction. The virus-Polybrene solution was then used to replace the medium of the PG13 cells. The plates were then wrapped with film and centrifuged at 1000 g at 32°C for 2 hours. The transduced cells were returned to the incubator, and the media was replaced with fresh D10 after 24 hours. A second transduction could be done to enhance the transduction efficiency using the 72-hour viral supernatant collected from the PlatGP cells, following the same protocol. The transduced PG13 cells were trypsinized from the wells 48 hours after transduction and transferred to a T75 flask. Flow cytometry analysis was used two days after transduction to measure the transduction efficiency of the PG13 cell line. EGFR expression on the PG13 cells indicated successful transduction. APC-conjugated anti-human EGFR antibody (Biolegend) was added to the cells at a dilution of 1:200 and incubated at room temperature for 30 minutes. After two washes with PBS + 2% FBS, cells were ready for flow analysis. To confirm the co-

expression of EGFR and CAR on the cells, FITC-conjugated goat F(ab')² anti-mouse IgG (Fab')² (Abcam) was added at 1:100 dilution together with the EGFR staining for flow analysis. Typically, the PG13 cell lines with over 90% positivity for EGFR and CAR expression would be used for retrovirus production. A portion of the PG13 cells was frozen in freezing media made of 90% FBS and 10% DMSO for storage. To generate viral particles with high concentration, the supernatant was collected when the transduced cells reached 90% confluence. The PG13 supernatant was centrifuged at 1500 g (acceleration 9, deceleration 5) for 15 minutes to remove the floating cells and debris and transferred to the ultracentrifuge tubes. The viral supernatant was centrifuged by the Sorvall WX⁺ Ultracentrifuge (Thermo Fisher Scientific) at 26000 g for 2 hours at 4°C. The medium was discarded gently by pipetting without disturbing the bottom of the ultracentrifuge tube. The virus pellets were resuspended in 1-2 mL D10 medium and stored at -80°C until use.

2.3.2. Production of CAR-expressing lentivirus

293T cells (ATCC) is the very common cell line that can be used to package lentivirus. The 293T cell line was recovered from cryopreservation and cultured in D10 medium for 5 days before transfection. Cells were passaged at a ratio of 1:6 when they reached 90% confluence by trypsinization. On the day before transfection (Day 0), 293T cells were seeded at 80-90% confluency to the plate. On the day of transfection, the medium was changed to DMEM+10%FBS (without antibiotics) and incubate for 1-2 hrs.

The PEI Transfection System:

Tube-1: 200 μ L of opti-MEM + 36 μ L of PEI transfection reagent, mixed gently but thoroughly.

Tube-2: 200 μ L of opti-MEM + expression plasmid + PSPAX2 + VSVG, mixed thoroughly.

Expression plasmid: PSPAX2: VSVG = 6:4:2

Tube-1 was combined with Tube-2 and let stand at room temperature for 20 minutes. Slowly added the complex to the culture dish drop by drop and transfected for 6 hours. The medium was replaced with pre-warmed 8 mL of complete D10 culture medium after 6 hours and returned to the incubator for further cultivation. The viral supernatant was collected at 48 hours and 72 hours. To remove cell debris, the supernatant was centrifuged at 1500 g (acceleration 9, deceleration 5) for 15 minutes at 4°C. Depending on the research needs, the viral supernatant was concentrated by adding 1/10 volume of 4M NaCl (Sigma-Aldrich) and 1/4 volume of 50% PEG6000 (Sigma-Aldrich). If the total volume of virus liquid exceeds 40 mL, this was divided it into two separate tubes. For example, for 36 mL of virus supernatant, add 3.6 mL of NaCl and 9 mL of 50% PEG6000. The solution was gently mixed by inverting the tube. Let the mixture stand at 4°C for 2 hours, inverting it every half hour. The mixture was then

centrifuged at 12500 g for 2 hours. The virus pellet was resuspended in the desired volume of medium and stored at -80°C until use.

2.3.3. Retrovirus and lentivirus titration

Virus titration was used to estimate the titer of retroviral particles produced by the PG13 packaging cells, which is a measure of the concentration of infectious viruses in the supernatant. 293T were passaged at a ratio of 1:6. One day before the transduction (Day 0), 293T cells were plated in a 6-well plate at a density of 1×10^5 cells/well in 2 mL of D10 medium and incubated at 37°C incubator overnight. If using freshly collected virus, floating cells and debris were removed through 1500 g centrifugation for 15 minutes. If using frozen virus, the retroviral aliquots were thawed on ice before use. On the next day (Day 1), prepared dilutions of the virus stock were titrated, as shown in Table 2.8. The D10 medium was gently aspirated, and 1.5 mL of a viral dilution was added to each well. Each well got one dilution with one well left over. The number of 293T cells was measured in the remaining wells for calculating the titer. The plates were incubated at 37°C for another 48 hours. The cells were trypsinized from the plates and stained with anti-human EGFR antibody for flow analysis. The percentage of EGFR-positive cells was recorded at each dilution.

The TU/mL (transducing units per millilitre) of virus was calculated as follows:

$$\text{TU/mL} = (\text{Number of cells transduced} \times \text{Percent fluorescent}) / (\text{Volume of virus added to each well in mL}).$$

Table 2.8. Virus titration

Dilution	Volume of retrovirus supernatant (μL)	Volume of D10 medium (μL)
1:10	150	1350
1:25	60	1440
1:50	30	1470
1:75	20	1480
1:100	15	1485
1:200	7.5	1492.5
1:2000	0.75	1499.25
1:3000	0.5	1499.5

2.3.4. Viral transduction of primary human T and MAIT cells

Human PBMCs could be obtained from fresh blood or frozen aliquots. To activate T cells, Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher Scientific) were mixed with PBMCs in R10 medium to achieve a bead-to-cell ratio of 1:1, and the cells were plated at a density of 1×10^6 /mL in a six-well plate. 10 ng/mL human IL-7 (Miltenyi Biotech) and IL-15 (Miltenyi Biotech) was added to the cells to support T cell activation. While human MAIT cells could be activated through different strategies. In the purified MAIT cell assay, MAIT cells were isolated by MACS as mentioned in section 2.1.5. After isolation, MAIT cells were activated in the similar way as the conventional T cells by the pan-T cell activator CD3/CD28 Dynabeads (Thermo Fisher Scientific) and 20 ng/mL human IL-12 (Miltenyi Biotech) and IL-18 (Miltenyi Biotech) for 48 hours before transduction. In PBMC assay, 30 million of PBMCs were cultured in the T25 flask. MR1 tetramer-loaded beads (at 1:1 bead-to-cell ratio) were added directly into the PBMC culture to allow the artificial APCs to present the antigen to the MAIT cells. 20

ng/mL IL-12 and IL-18 were added as supplement and replenished every 2 to 3 days. Either retroviral or lentiviral transduction was conducted 48 hours after cell activation.

RetroNectin is a recombinant human fibronectin fragment, which can be used to enhance the binding of retroviral particles to T cells. To prepare for viral transduction, RetroNectin-coated plates were prepared by adding 2 mL of PBS containing 15 µg of RetroNectin (Takara Bio) to a well of a non-tissue culture treated 6-well plate and stored in the 4°C refrigerator overnight (Day 0). On the day of gene transfer (Day 1), unbound RetroNectin was removed by PBS wash and the plate was blocked by 2% BSA in PBS at room temperature for 30 minutes. Once the wash solution is removed, the plate is ready for use. The RetroNectin-coated plate could be preserved at 4°C for a maximum of one week. Viral supernatant (MOI=10) was added to each well and then centrifuged at 2000 g at 32°C for 2 hours. During centrifugation, activated PBMCs were collected, and the number of viable cells was determined. As the culture medium could influence gene transfer efficiency, the cells were resuspended in the original R10 medium with fresh cytokines at a density of 1×10^6 /mL. After centrifuging the RetroNectin-coated plate with virus, the supernatant was aspirated, and the wells were washed with PBS. 1 mL of cell suspension was added to each well and centrifuged at 300 g for 10 minutes. The plate was then returned to the incubator. Two days after gene transfer (Day 4), transduction efficiency was analysed by flow cytometry through EGFR expression. The transduced CAR-T cells were cultured in R10 medium containing cytokines until they were required for further experiments. The cells were counted and suspended at a concentration of 0.5 to 1 million cells/mL in fresh R10 medium with cytokines every 2-3 days. The T cells were cultured *in vitro* for up to 14 days. If transduction efficiencies were below 50%, CAR expressing cells were enriched by staining with biotinylated EGFR antibody (Biolegend) followed by positive

selection with streptavidin microbeads (Miltenyi Biotech). CAR-T cells were frozen in freezing media containing 90% FBS + 10% DMSO in the liquid nitrogen for long-term storage.

2.4. Phenotypic and functional assay for T cells

2.4.1. Flow cytometry of transduced cells and target cells

Table 2.8 lists the antibodies employed to label T cells and MAIT cells. The cells were harvested and washed with PBS through centrifugation at 500 g for 5 minutes. They were then suspended in 200 μ L PBS containing antibodies at a specific concentration and incubated in the dark at room temperature for 30 minutes. The cells were washed with five to ten volumes of PBS and then centrifuged at 500 g for 5 minutes. After resuspending the cells with 300 μ L PBS, flow cytometry analysis was conducted using a BD LSR II instrument (BD Biosciences). Prior to the run, the LSR II was calibrated with CST beads (BD Biosciences) to ensure standardization of fluorescent analysis. Data acquisition was carried out using FACSDiva version 6.0 software (BD Biosciences) and saved as FCS files. Flow cytometry data obtained from the BD LSR II were analysed using FlowJo version 10.4.0 software (FlowJo, USA).

Cells with successful CAR transduction were identified by positive EGFR staining, which is incorporated in the CAR construct and co-expressed in the transduced cells. Validation of CD19 antigen expression on the target B cell line was also conducted through flow cytometry analysis, employing a staining protocol akin to the one described above, with the utilization of an anti-human CD19 antibody conjugated with Brilliant Violet 510 (Biolegend) at a dilution of 1:200. Depending on the research requirements and the desired colour panel, either DAPI

(Miltenyi Biotec) or the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific) was utilized to differentiate live and dead cells, following the manufacturer's instructions.

Table 2.9. List of Antibodies used for staining transduced T and MAIT cells.

Target	Clone	Conjugation	Manufacture	Dilution
Mouse anti-human CD3	REA613	FITC	Miltenyi Biotec	1:200
Mouse anti-human CD161	DX12	APC	BD	1:200
Mouse anti-human TCR V α 7.2	3C10	PE	Biolegend	1:200
Mouse anti-human EGFR Antibody	AY13	APC	Biolegend	1:200
Mouse IgG F(ab) $'_2$	Polyclonal	Alexa Flour 488	Jackson ImmunoResearch	1:50
Mouse anti-human CD4	SK3	APC	BD	1:200
Mouse anti-human CD8	SK1	PerCP-Cy5.5	BD	1:200
Mouse anti-human CD45RO	UCHL1	FITC	Biolegend	1:200
Mouse anti-human CD45RA	HI100	APC	Biolegend	1:200
Mouse anti-human CD62L	DREG-56	PE	BD	1:200
Mouse anti-human CD195 (CCR5)	2D7	BV421	BD	1:200
Mouse anti-human CD196 (CCR6)	11A9	BV711	BD	1:200
Mouse anti-human CD197 (CCR7)	G043H7	PE-Cy7	Biolgend	1:200

2.4.2. Cytotoxicity assay

Non-radioactive killing assay was performed to evaluate the killing efficacy of CAR-modified cells. In brief, 5 million of the target cells were stained with 1 μ M CFSE (Biolegend) in 5 mL PBS for 15 minutes at 37°C, protected from light. Five times of the original staining volume of culture medium (containing at least 1% FBS) was added to the cells and incubate for 10 minutes to remove any free dye remaining in the solution. The CFSE-labelled cells were incubated for at least 10 minutes before the co-culture to allow CFSE to undergo acetate hydrolysis. After being washed with PBS for three times, the CFSE-labelled target cells were mixed with the effector CAR-T cells in ratio at 1:1, 1:5. The 200 μ L co-culture was incubated for 20 hours in the incubator at 37°C. 1 μ L DAPI and 5 μ L Countbright beads (BD Biosciences) were added to the samples. The samples were acquired by flow cytometry at constant speed (medium) and 5000 beads were acquired to quantify the surviving target cells. The gating strategy is shown in Figure 2.2. The number of surviving target cells was calculated as: Cells in tube = (cells collected/ beads collected) x total beads added to the tube.

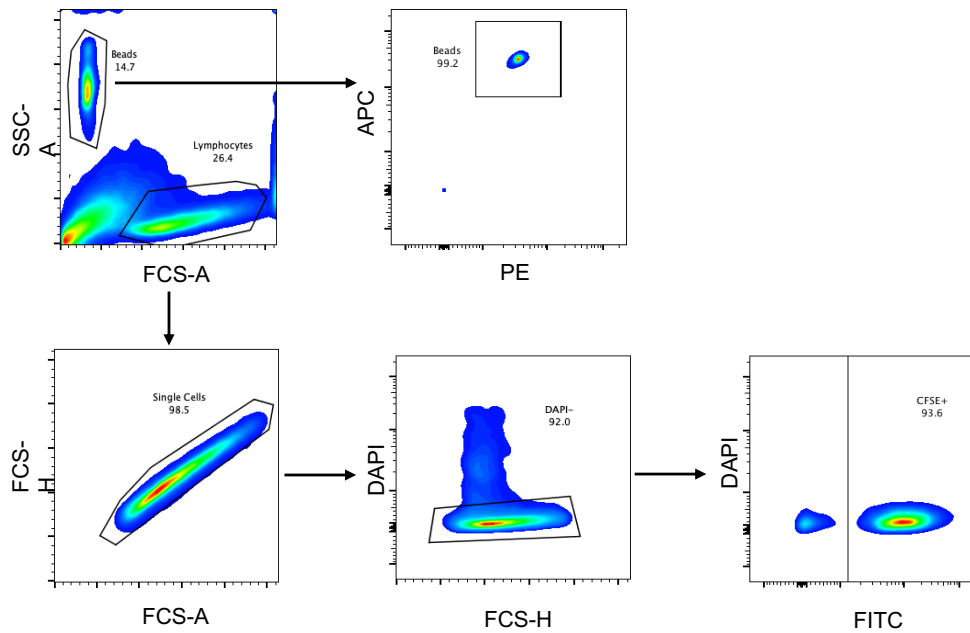


Figure 2.2. Gating strategy for flow cytometry analysis of CAR-T cytotoxicity.

The CountBright beads were gated by SSC and FSC, followed by the second gating with PE and APC channels to further exclude non-CountBright-beads noise. Surviving target cells were firstly gated on lymphocyte and then single cells. Then the dead cells were excluded by gating on DAPI. The remaining CFSE⁺ cells represented surviving target cells after co-culturing. The numbers of CFSE⁺ cells at 0 hour were used as the baseline to estimate the killing efficacy of the effector cells. Flow cytometry analysis carried by FlowJo.

2.4.3. Mixed Lymphocyte Reaction (MLR) assay

MAIT cell and T cell proliferative responses to autologous and allogeneic cells *in vitro* were measured by the two-way MLR, where lymphocytes derived from different donors act as both responders and stimulators against each other. Human PBMCs from different donors were stained with CFSE following a similar protocol mentioned above. Then the CFSE-labelled PBMCs ($10^6/\text{mL}$) were cultured with allogeneic PBMCs at a 1:1 cell ratio. Alloreactive responder MAIT and T cells were identified at the end of the six days culture by gating on lymphocytes following exclusion of dead cells. MAIT cells were distinguished from the other T cells by the staining of MR1-tetramer. The proliferating responders were identified by gating on the CFSE dim or negative cells after gating out CFSE high non-proliferating.

2.5. Statistical Analysis

Statistical analysis was performed using GraphPad Prism software version 8.0. A two-tailed unpaired Student's t-test was used to compare data between two groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. One-way ANOVA was used to compare data among multiple groups. * $P < 0.05$, ** $P < 0.01$. All the data with error bars are presented as mean values $\pm$ standard error of the mean (SEM). A P value of less than 0.05 was considered significant.

Chapter 3. Design and optimised production protocol for CD19-specific CAR cells *in vitro*

The success of CAR-T cell manufacturing and their demonstrated therapeutic efficacy in hematologic malignancies have laid the groundwork for the exploration of alternative T cell subsets as novel CAR backbones. The first part of this thesis is dedicated to the development, optimization, and establishment of protocols for CAR production, serving as a foundational platform for CAR-MAIT cell generation. This chapter encompasses the construction of a third generation anti-CD19 CAR, the establishment of viral transduction platforms, and the validation of the feasibility of generating CAR-MAIT cells.

3.1. Cloning of CD19-specific CAR constructs

The CAR targeting CD19 were engineered to redirect T cell specificity. These CAR designs incorporate the scFv derived from the monoclonal IgG antibodies FMC63, which has well-documented specificity for human CD19 and utilized to create the scFv component of CARs in the treatment of diseases like B cell lymphomas and leukaemia (Kochenderfer et al., 2009). The DNA sequences of the antibody FMC63 with the specificity towards human CD19 were found from the public patent (US6410319B1). The DNA sequences and the corresponding amino acids of the VL chain and VH chain of the FMC63 were shown in Figure 3.1 A and B respectively. The VL and VH were connected through a (GGGS)₄ linker, which confers the proper flexibility to scFv.

A	DNA sequence of VL FMC63:	DNA sequence of VH FMC63:
	GACATCCAGATGACACAGACTACATCCTCCCTGTCTGCCTC TCTGGGAGACAGAGTCACCATCAGTTGCAGGGCAAGTCA GGACATCTCTAAGTATTGAATTGGTATCAGCAGAAACCAG ATGGAAGTGTAACTCCTGATCTACCATACATCAAGATTAC ACTCAGGAGTCCCATCAAGGTTCACTGGCAGTGGGTCTGG AACAGATTATTCTCTACCATTAGCAACCTGGAGCAAGAAG ATATTGCCACTTACTTTTGCCAACAGGGTAATACGCTTCCGT ACACGTTTCGGAGGGGGGACTAAGTTGGAAATAACA	GAGGTGAAAGTGCAGGAGTCAGGACCTGGCCTGGTGGCG CCCTCACAGAGCCTGTCCGTACATGCACTGTCTCAGGGGT CTCATTACCCGACTATGGTGAAGCTGGATTCCGCAGCCTC CACGAAAGGGTCTGGAGTGGCTGGGAGTAATATGGGGTA GTGAAACACATACTATAATTCACTCTCAAATCCAGACTG ACCATCATCAAGGACAACCTCAAGAGCCAAGTTTCTTAAA AATGAACAGTCTGCAAAGTATGACACAGCCATTACTACT GTGCCAAACATTATTACTACGGTGGTAGCTATGCTATGGACT ACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAGCGGC CGCA
B	Amino acid sequence of VL FMC63:	Amino acid sequence of VH FMC63:
	DIQMTQTSSLSASLGDRVTISCRASQDISKYLNWYQKPDG TVKLLIYHTSRLHSGVPSRFSGSGSDYSLTISNLEQEDIATYF CQQGNTLPYTFGGGKLEIT	EVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPR KGLEWLVGIWGSSETTYNSALKSRITIKDNSKSVFLKMNSL QTDDTAIYYCAKHYGGSYAMDYWGQGSVTVSSAAA

Figure 3.0.1. Nucleic acid sequences and amino acid sequences of the VL chain and VH chain of anti-CD19 monoclonal antibody FMC63.

(A) The nucleic acid sequences of VL (321 bp) and VH (369 bp) of FMC63. (B) The amino acid sequences of VL (107 aa) and VH (123 aa) of FMC63.

In this thesis, a third-generation CAR construct was constructed due to the recent research findings that highlight its superior performance when compared to the earlier first- and second-generation CARs (Enblad et al., 2018; Shadman et al., 2020). The CAR design comprises a CD8 transmembrane domain, CD28 and 4-1BB endodomains for co-stimulatory signaling, and the CD3 ζ chain for activation. In addition, a truncated EGFR (tEGFR) is tagged behind CAR and connected by the self-cleaving 2A linker, serving a dual purpose within the CAR structure (Figure 3.2 A and B). It serves as a tracking marker and selection gene, facilitating the detection of CAR expression levels on the cells. Additionally, the incorporation of tEGFR offers an external mechanism for modulating CAR-T cell function. In cases where unexpected side effects or toxicities emerge, cetuximab can be administered to deplete engineered CAR-T cells through antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC), thereby providing an additional layer of safety control (Wang et al., 2011).

To construct the anti-CD19 CAR-expressing vector, the synthesized scFv fragment of FMC63 underwent cleavage by *NcoI* and *NotI* restriction enzymes, producing compatible overhangs for subsequent ligation. The resulting fragment was then inserted into the MSCV backbone template between the corresponding restriction enzyme sites (Figure 3.2 C). Validation of the cloned cassettes within the vector was conducted through sanger sequencing.

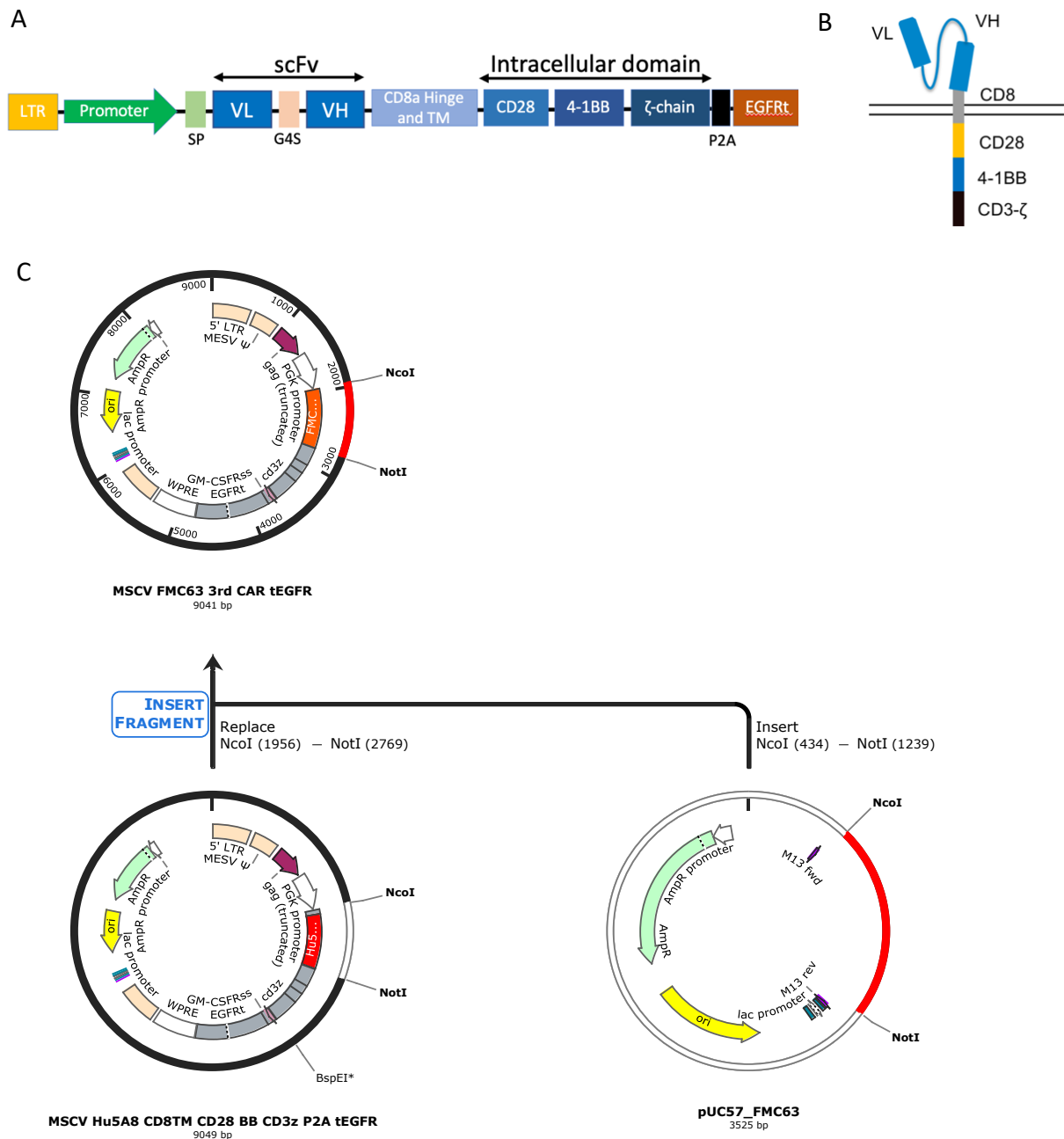


Figure 3.2. Schematic representation of the CAR construct.

(A) The schematic diagram represents the functional elements included in the CAR construct. The scFv derived from monoclonal antibody FMC63 (anti-CD19) was fused with a CD8 transmembrane domain (TM) followed by CD28 endodomain, 4-1BB and CD3 ζ intracellular domains. EGFR was tagged behind CAR via self-cleaving 2A linkers. SP: Igk chain leader sequence; ScFv: single-chain variable fragment; TM: transmembrane motif. (B) Schematic diagram of the chimeric CAR protein expressed on T cell surface. (C) Overview of the CAR-expressing vector construction. The MSCV-Hu5A8-EGFRt vector was linearized by restriction enzyme digestion with *NcoI* and *NotI*. The synthesized FMC63 scFv fragments were also cleaved by the same restriction enzymes to generate compatible overhangs and ligated to the target vector by T4 ligase. Figure adapted from Ma, 2019 and SnapGene.

3.2. Establishing CAR-expressing retrovirus platform

In CAR-T cell production, the choice of transduction platform plays a pivotal role in determining the efficiency and dynamics of CAR expression. The establishment of robust retrovirus transduction platforms, characterized by the engineering of CAR-expressing retroviral packaging cell lines, offers a pathway to achieve sustained and long-term production of viral supernatant for CAR transduction. On the other hand, the lentivirus transduction platform provides a transient yet highly versatile method for generating CAR-expressing viruses to transduce both dividing and non-dividing cells. These two platforms contribute uniquely to the evolving landscape of CAR-T cell therapy, offering promising solutions for achieving successful CAR expression in T cells. In this thesis, both retroviral and lentiviral transduction platforms have been established to determine the optimal way to generate CAR-expressing virus with high transduction efficiency.

In the initial phase of this study, a stable retrovirus-packaging cell line utilizing the PG13 cell line was established to ensure consistent production of CAR-expressing viruses. Retroviral vector infection, known for its safety and effectiveness in gene transfer, facilitates the stable expression of the gene of interest in target cells. Plat-GP and PG13, widely employed retroviral packaging cell lines, are involved in generating retroviral vectors for CAR-T therapy. A two-step transduction protocol was employed to enable the reliable production of high-titer retroviral vectors (Figure 3.3). In brief, Plat-GP cells underwent transfection with the retroviral vector containing the desired anti-CD19 CAR gene and VSV-G envelope vector as the essential viral packaging gene. The transfected Plat-GP cells only act as a transient retrovirus packaging cell line and the retroviral supernatant was then harvested to transduce

PG13 cells, making them as a stable retroviral packaging cell line. The transduced PG13 cell line was able to continuously and stably produce large quantities of CAR-expressing retrovirus for CAR-T cell therapy.

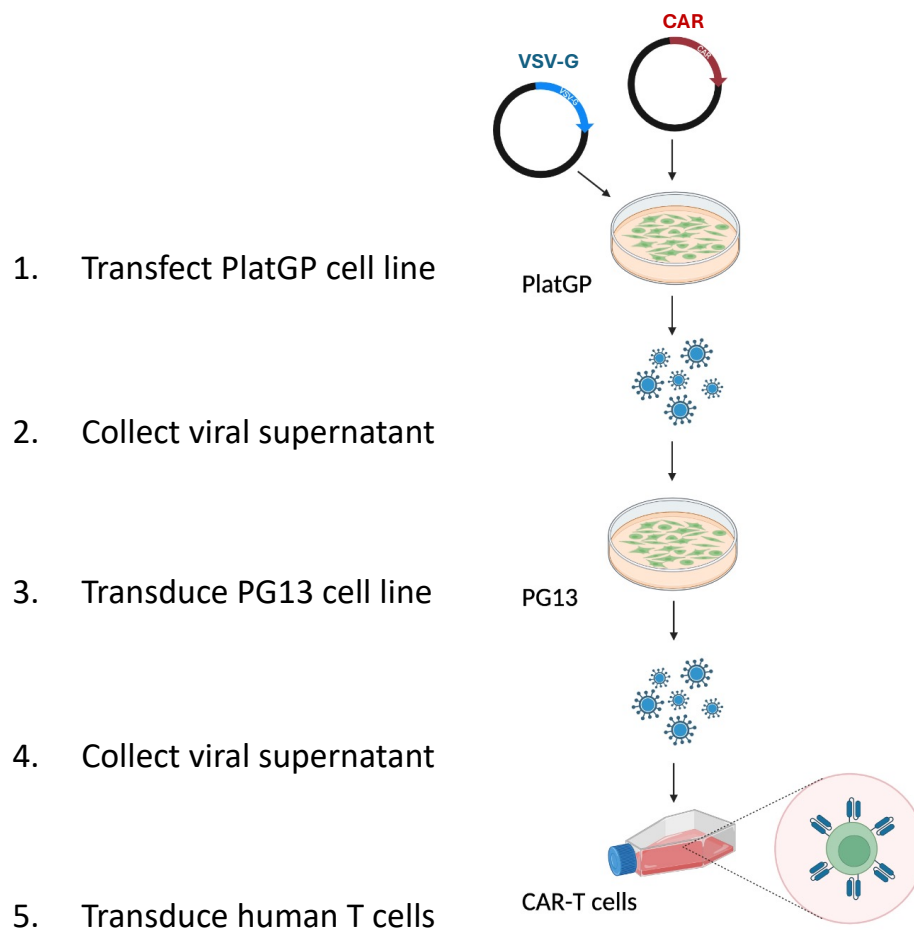


Figure 3.3. Flow chart representation of the two-step retrovirus transduction protocol.

Two-step transduction protocol is an efficient method of gene transfer using retroviral vectors: (1) production of the retroviral particles in Plat-GP packaging cells; (2) infection of stable retrovirus packaging PG13 cells with the retroviral particles. The CAR-encoding retroviral particles produced by PG13 cells can then be used to transduce T cells for subsequent CAR-T cell therapy. Figure modified using BioRender.

The efficiency of Plat-GP cell transfection and PG13 cell transduction was examined to ensure the maximum production of retrovirus for CAR gene transfer. According to the double staining of the mouse scFv-specific anti-mouse IgG F(ab')₂ antibody in combination with the EGFR-specific antibody, the expression levels of EGFR and CAR were tightly correlated, validating the effectiveness of EGFR as the CAR detection marker (Figure 3.4 A). The supernatant containing retroviral particles was collected from transfected Plat-GP cells with over 90% efficiency and used for subsequent PG13 cell transduction to produce the stable virus packaging cell line. The transduction efficiency on PG13 cells reached approximately 100% EGFR positive rate circa 48 hours post-transduction (Figure 3.4 B). This implies that effective gene delivery can be accomplished without the need for selecting single PG13 cell cloning for experimental use. The harvested retroviral supernatant from the transduced CAR-expressing PG13 cell lines would be applied for CAR-T and CAR-MAIT cell transduction in the later section.

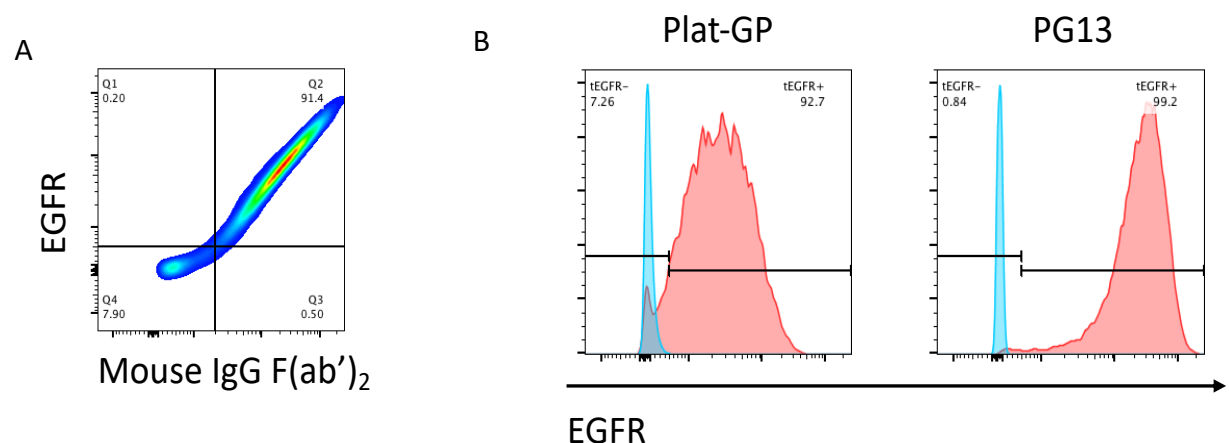


Figure 3.4. Flow cytometry analysis of CAR gene transfer efficiency by retrovirus.

(A) Transduced PG13 cells were stained by anti-EGFR antibody and anti-mouse IgG F(ab')₂ antibody for examining the co-expression level of EGFR and CAR. (B) Virus packaging cell lines Plat-GP (left) and PG13 (right) were transduced with anti-CD19 CAR expressing plasmid respectively. The expression of EGFR was stained by the anti-EGFR antibody for flow cytometry analysis. The blue histogram indicated the non-transduced cells as the negative control while the red histogram represented the expression level of EGFR on the transduced cells.

3.3. Establishing CAR-expressing lentivirus platform

In addition to employing the retrovirus system for CAR transduction, a complementary lentivirus platform has been implemented in a later phase of the study, serving as a versatile alternative avenue for gene delivery. Lentivirus transduction exhibits a distinct capability to transduce non-dividing and slowly dividing cells, enhancing the adaptability of the transduction process. This versatility makes it a preferred choice in the context of CAR-T cell therapies, guaranteeing high-efficiency gene delivery and safe expression of CAR constructs in the target cells. Development of the anti-CD19 lentiviral vector was based on the existing anti-CD19 CAR-expressing retroviral plasmid. The sequence of the anti-CD19 CAR construct, encompassing crucial elements such as the Igk leader sequence, FMC63 scFv region, CD8 transmembrane domain, CD28 endodomain, 4-1BB and CD3 ζ intracellular domains, and EGFR, was extracted from the retrovirus vector through PCR amplification. During this process, the amplified products acquired compatible overhangs for the *SbfI* and *BstXI* restriction enzymes, facilitating successful insertion into the linearized lentivirus template vector LA-05-2 through In-fusion cloning (Figure 3.5). The integrity of the cassettes integrated into the lentiviral vector was subsequently verified through sanger sequencing. To produce lentivirus, 293T cells underwent transfection with the CAR-expressing lentivirus plasmid, alongside multiple essential viral packaging genes including VSV-G and PSPAX2. These additional elements are instrumental in facilitating efficient lentiviral packaging and ensuring the production of high-titer virus particles. The production of lentivirus involved harvesting the viral supernatant at 48 hours and 72 hours post-transfection, maximizing the yield of viable viral particles. The collected lentiviral supernatants were mixed and used as a critical resource for subsequent CAR transduction (Figure 3.6).

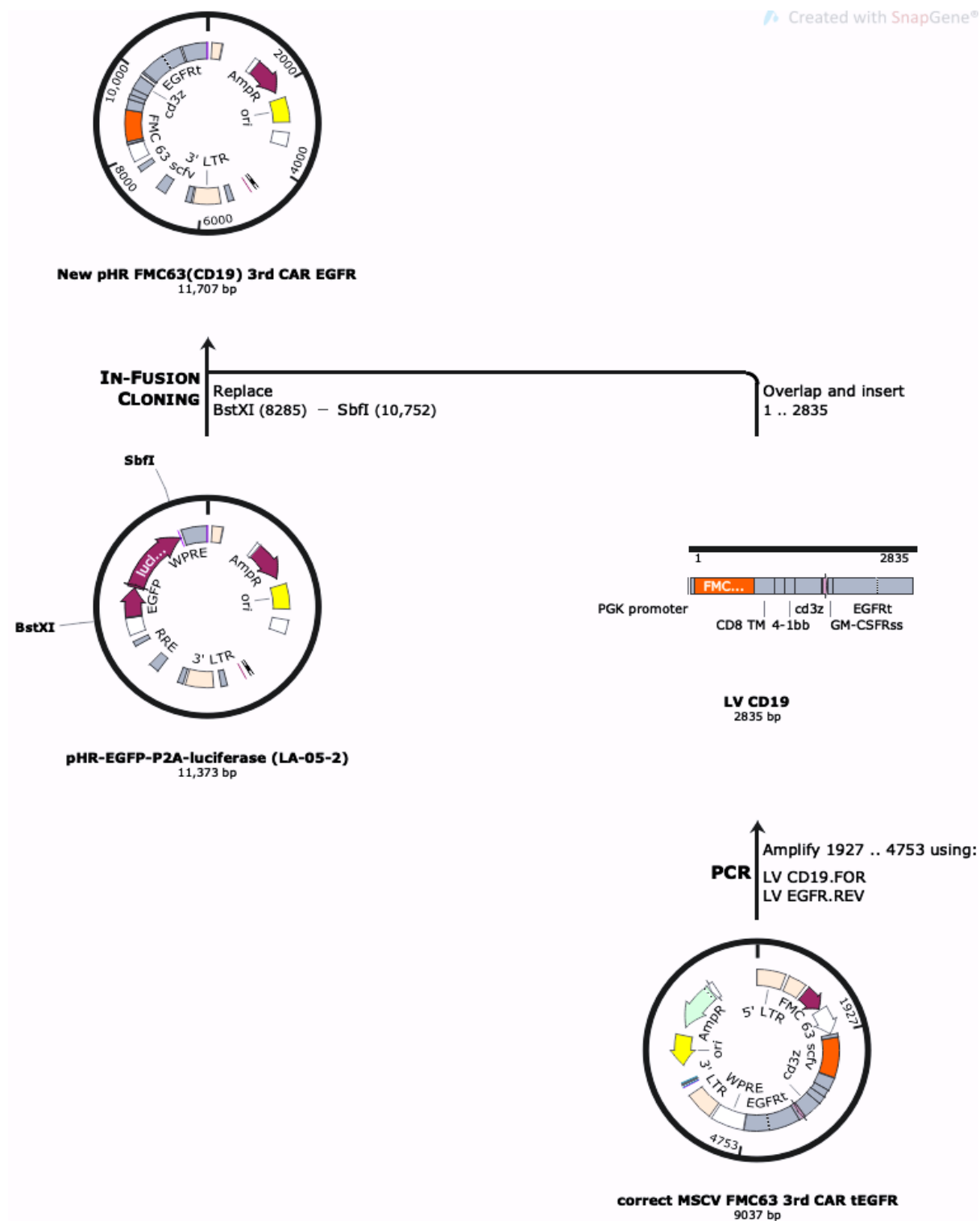


Figure 3.5. Cloning of the 3rd generation CAR construct into the lentivirus vector.

The CAR constructs, including the Igk chain leader sequence, FMC63 scFv, a CD8 transmembrane domain, CD28 endodomain, 4-1BB and CD3 ζ intracellular domains and EGFR, were amplified by PCR from the retrovirus vector. The lentivirus template vector LA-05-2 was linearized by restriction enzyme digestion with *SbfI* and *BstXI*. The purified PCR products were cloned into the target lentivirus vector by In-Fusion cloning. Figure adapted from SnapGene.

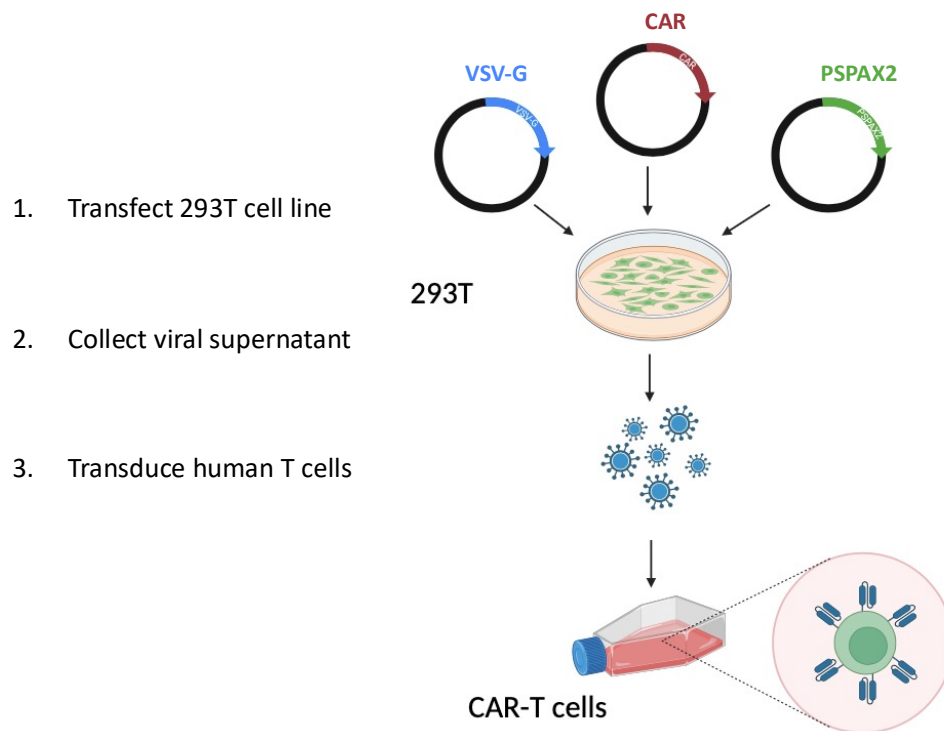


Figure 3.6. Flow chart representation of the lentivirus transduction protocol.

Transient production of lentivirus involves (1) production of the lentiviral particles in 293T cells using CAR-expressing plasmid, VSVG and PSPAX2 plasmids; (2) Lentivirus supernatant was collected at 48 hours and 72 hours after transfection. The CAR-encoding lentiviral particles produced by 293T cells could then be used to transduce T cells for subsequent CAR-T cell therapy. Figure modified using BioRender.

The transfected 293T cells were collected and analysed using flow cytometry. The transfection efficiency of 293T cells approached nearly 100%, as evidenced by the high percentage of cells positive for EGFR expression after 72 hours (Figure 3.7 A). These results indicate the successful and robust delivery of the gene using this methodology.

Viral titration experiment was then performed to estimate the titer of both retroviral and lentiviral vector in TU/mL. Each viral stock was diluted at different ratios to transduce 293T cells. Here, lentivirus produced via transfection of 293T cells exhibited a superior virus titer compared to retrovirus generated through transduced PG-13 cells. For instance, the retrovirus achieved only approximately 30 to 40% transduction efficiency at a 1:200 dilution. In contrast, lentivirus demonstrated a remarkable performance, surpassing 60% transduction efficiency under similar conditions (Figure 3.7 B). To determine the precise titer of the lentivirus, additional dilutions were performed until reaching a 1:3000 dilution, where the transduction efficiency of the lentivirus in 293T cells achieved at approximately 40% (Figure 3.7 C). The retrovirus attained a titration of approximately 2.8×10^6 TU/mL, whereas the lentivirus demonstrated a significantly higher titer at 5.4×10^7 TU/mL. This discrepancy underscores the enhanced potency and efficacy of lentivirus, substantiating their viability and superiority for gene delivery applications.

Hence, to facilitate successful and efficient CAR-T and CAR-MAIT cell production and subsequent pre-clinical evaluation, robust methodologies for CAR gene delivery have been devised to generate high virus titers. The strategic establishment of both the lentivirus platform and retrovirus system represents a methodological expansion that leverages the distinctive strengths of each system. The retrovirus system enables the establishment of a

stable CAR-expressing virus packaging cell bank, ensuring consistent production of retrovirus with both quality and titers suitable for CAR transduction within a concise ten-day period. In parallel, the lentivirus platform exhibits broader cell tropism, infecting both dividing and non-dividing cells, in contrast to retroviruses, which can only infect dividing cells. This broader cell tropism of lentiviruses allows for more efficient transduction of target cells, including T cells, which may be in a non-dividing state. Besides, the lentivirus platform demonstrates a higher virus titer, facilitating efficient CAR-T transduction. However, it is important to note that while the lentivirus platform excels in transducing cells within a shorter timeframe with high efficiency, its transient one-time production of the virus requires repeated cell transfections and substantial amounts of transfection reagents. The adoption of both system approaches not only enhances the overall versatility and adaptability of CAR transduction but also positions the methodology to effectively address diverse clinical scenarios and therapeutic requirements.

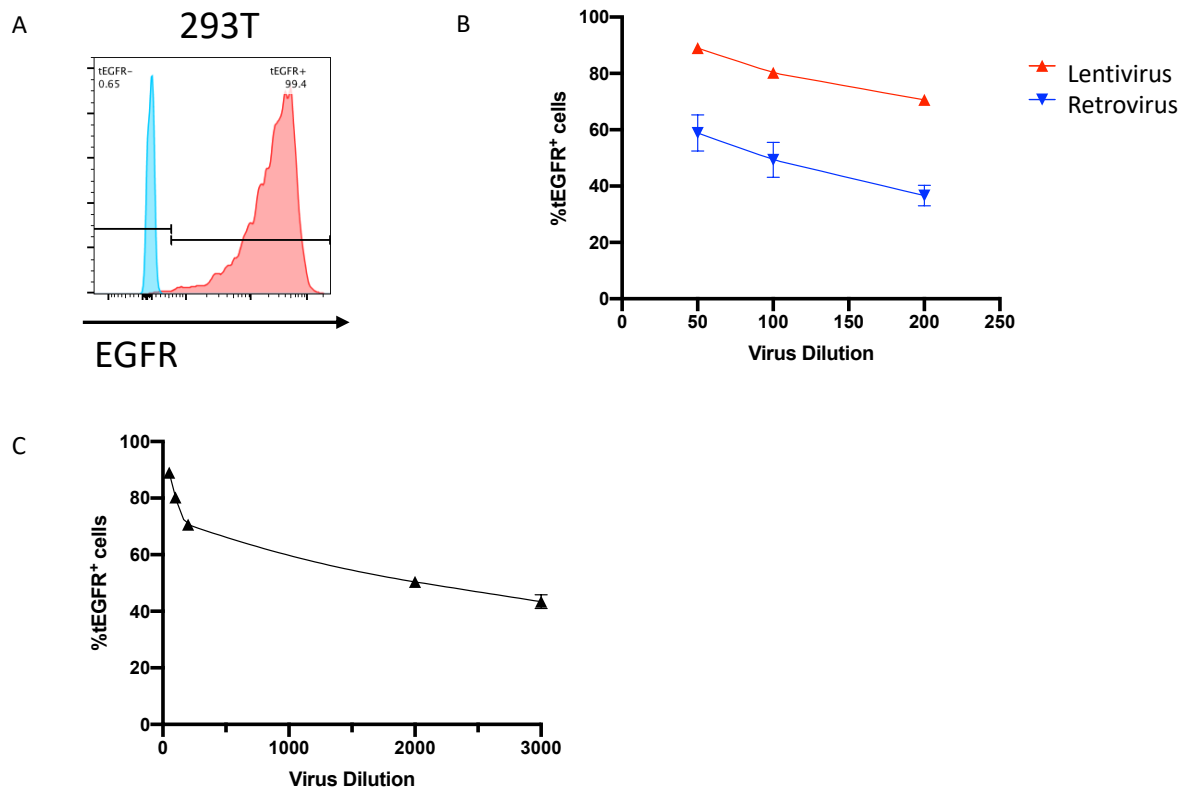


Figure 3.7. Flow cytometry analysis of CAR gene transfer efficiency by lentivirus.

(A) Virus packaging cell line 293T was transduced with anti-CD19 CAR expressing plasmid. The expression of EGFR was stained by the anti-EGFR antibody for flow cytometry analysis 72 hours post-transfection. The blue histogram indicated the non-transduced cells as the negative control while the red histogram represented the expression level of EGFR on the transfected cells. (B) The retroviral supernatant (blue line) and lentiviral supernatant (red line) underwent titrations for accurate determination of viral titers. In the case of retrovirus, a dilution of 7.5 μ l retrovirus into 1500 μ l medium yielded an approximate 30-40% transduction efficiency in 0.5×10^5 cells, representing a titration of approximately 2.8×10^6 TU/mL. Lentivirus achieved around 70% transduction even at a 1:200 dilution. Further titration was required for precise virus titer calculation. (C) Lentiviral particles were further diluted, reaching approximately 40-50% transduction efficiency in 0.5×10^5 cells at a 1:3000 dilution. The titration indicated the lentivirus titer at roughly 5.4×10^7 TU/mL. Data with error bars represent mean $\pm$ SEM. Each experimental condition was tested using two replicates within a single experiment. Results shown are derived from two independent experiments (n=2).

3.4. Expression of CD19-specific CAR on primary human T and MAIT cells

To facilitate the introduction of CAR into primary human T cells and MAIT cells, the target immune cells must undergo activation before transduction. A common approach to activate T cells from PBMCs involves utilizing Dynabeads Human T-Activator CD3/CD28 along with IL-7 and IL-15 for a duration of 2-3 days. MAIT cells were identified as CD3⁺ Vα7.2⁺ CD161⁺ or CD3⁺ MR1 tetramer⁺ lymphocytes (Figure 3.8). However, given that MAIT cells represent only a small proportion of circulating T cells in human blood, a purification assay was conducted to isolate MAIT cells from PBMCs before activation through MACS. In this process, MAIT cells in PBMCs were specifically recognized and purified using PE-conjugated 5-OP-RU-loaded MR1 tetramer and anti-PE microbeads. Following separation, more than 90% of the remaining cell population comprised MAIT cells (Figure 3.8), making them suitable for subsequent cell activation. Similar as the conventional T cells, the purified MAIT cells were stimulated by CD3/CD28 Dynabeads and cytokines IL-12 and IL-18 for activation as described in the Materials and Methods section 2.1.6.

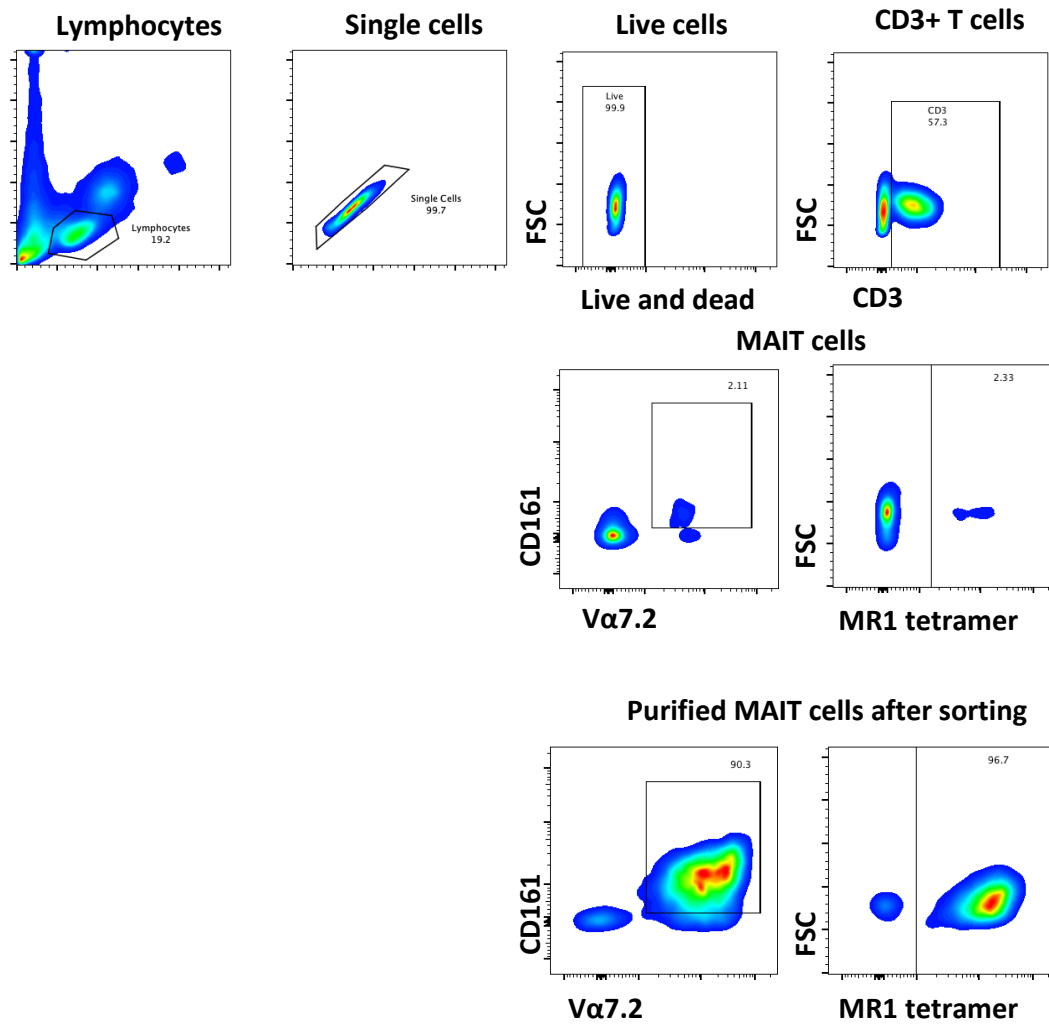


Figure 3.8. Gating strategy of MAIT cells in PBMCs and purified MAIT cells after MACS sorting.

Human MAIT cells were identified as CD3⁺ Vα7.2⁺ CD161⁺ or CD3⁺ MR1 tetramer⁺ lymphocytes. They typically constitute 0.1% to 10% of total T cells in human blood. In the purified assay, MAIT cells can be isolated from PBMCs using MACS, achieving a purity exceeding 90%.

Given that MAIT cells also express CD3 on their surface, there is a suspicion that the general T cell activator CD3 might effectively activate unconventional MAIT cells similarly to conventional T cells. Following three days of stimulation with CD3/CD28 Dynabeads, MAIT cells exhibited a clustering pattern like the conventional CD8⁺ T cells. Examination of activation markers revealed a robust upregulation of CD25 and CD69 in both MAIT cells and CD8⁺ T cells. However, CD8⁺ T cells exhibited a higher level of upregulation in CD38 and HLA-DR expression compared to MAIT cells (Figure 3.9). The observed similarities and differences in the expression of activation markers between MAIT cells and conventional T cells offer intriguing insights into their activation dynamics. The shared upregulation of CD25 and CD69 suggests a commonality in the initial response to CD3/CD28 Dynabead stimulation, indicating a parallel initiation of early activation events in both cell types. This aligns with the notion that CD3 activation pathways are conserved across various T cell subsets, irrespective of their unconventional or conventional nature. In this sense, the CD3/CD28 Dynabeads could serve as a suitable method for activating MAIT cells before viral transduction. However, the subtle differences, such as the elevated expression of CD38 and HLA-DR in CD8⁺ T cells compared to MAIT cells, raise questions about potentially distinct downstream signaling cascades. CD38 is associated with T cell activation and effector functions, while HLA-DR is a marker of antigen presentation and immune response modulation. Their co-expression reflects immune activation (Henry et al., 2018). The lower levels of CD38 and HLA-DR observed in MAIT cells may be rooted in the unique activation pathways characterizing these unconventional T cells. MAIT cells, with their specificity for microbial-derived vitamin B metabolites presented by MR1, might engage signaling cascades distinct from conventional T cells activated through CD3/CD28 stimulation. The nature of the activating ligands and the subsequent downstream signaling might orchestrate differential expression patterns of activation markers. The timing

and kinetics of activation-induced marker expression represent another facet of the observed differences. MAIT cells might exhibit a delayed or more transient upregulation of CD38 and HLA-DR, indicative of a temporally distinct response compared to conventional T cells. This temporal variation could reflect the unique kinetics associated with the MAIT cell activation process. Additionally, the sensitivity of MAIT cells to activation signals may differ from conventional T cells. The distinctive nature of MAIT cell antigen recognition and activation might render them less responsive to certain signals that drive high CD38 and HLA-DR expression in conventional T cells.

These findings may prompt further investigation into the specific signaling pathways and downstream responses triggered by CD3 activation in MAIT cells versus conventional T cells. Understanding the precise reasons for these differences would require detailed mechanistic studies investigating the signaling pathways and transcriptional regulation specific to MAIT cell activation. Further research in this direction could unravel the distinctive features of MAIT cell activation and contribute to our broader comprehension of their role in immune responses and potentially inform strategies for harnessing their therapeutic potential.

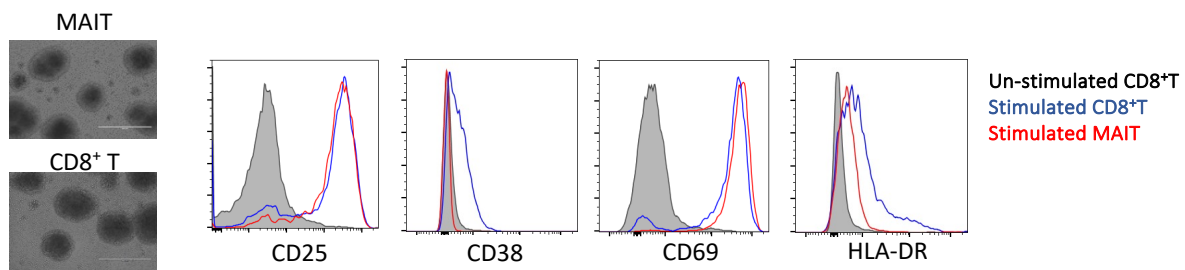


Figure 3.9. Activation of purified MAIT cells by CD3/CD28 Dynabeads.

After three-day stimulation with CD3/CD28 Dynabeads, both MAIT cells (upper panel) and CD8⁺ T cells (lower panel) formed cell clusters. Representative histogram overlays of activation marker, CD25, CD38, CD69 and HLA-DR, expression on unstimulated CD8⁺ T cells (grey histograms), stimulated non-MAIT CD8⁺ T cells (blue histograms) and stimulated MAIT cells (red histograms).

Following activation, T cells and MAIT cells underwent transduction using the viral supernatant. Initially, retroviral transduction was implemented in the study to deliver CAR genes. Retroviral transduction is a commonly used method for introducing genetic material into target cells, leveraging the ability of retroviruses to integrate their genome into the host cell's DNA. However, despite its effectiveness, retroviral transduction has limitations such as lower titers and limited transduction efficiency in non-dividing cells (Irving et al., 2021). Therefore, to overcome these limitations and achieve higher transduction efficiency, the study transitioned to employing lentiviral vectors in the later stages. As depicted in the representative graph, retroviral transduction resulted in a transduction rate of only 20-30% on T cells due to the low virus titer, whereas lentiviral transduction achieved a significantly higher transduction rate, reaching over 50-70% on the day of harvest (Figure 3.10).

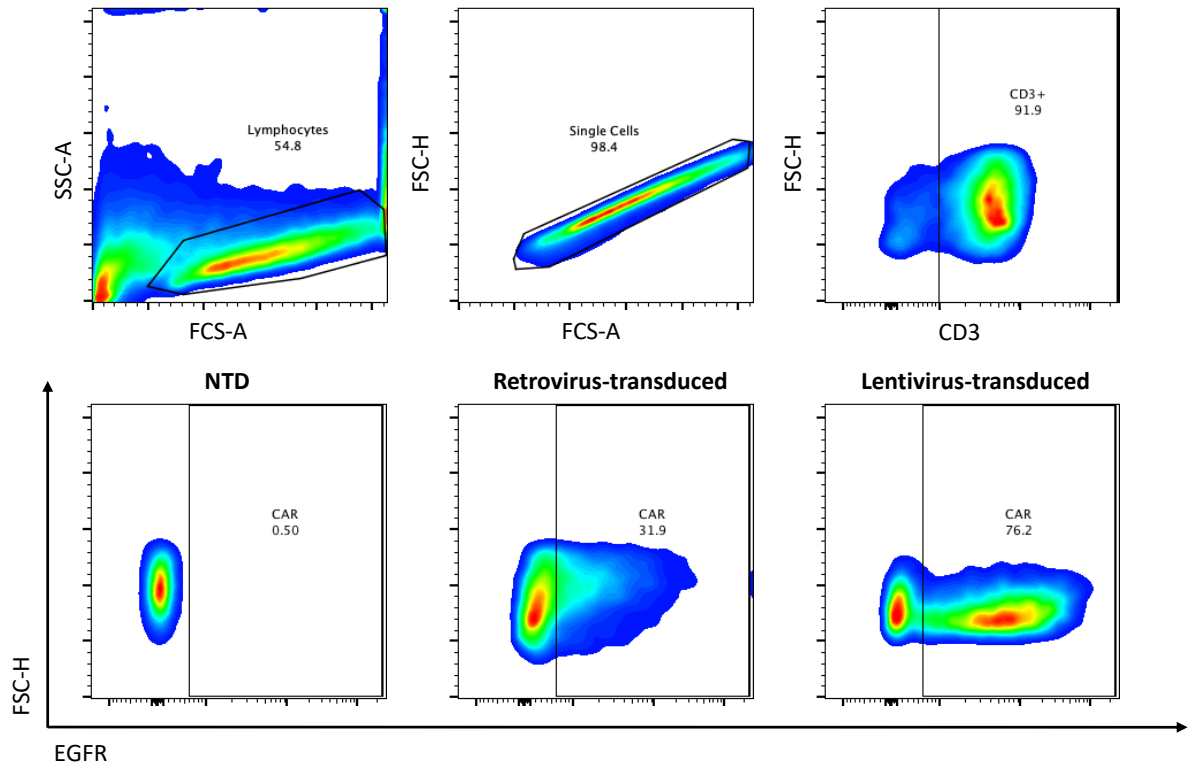


Figure 3.10. Transduction efficiency of retrovirus and lentivirus on T cells.

Activated T cells were transduced by retrovirus and lentivirus respectively. Cells were gated on CD3⁺ single lymphocytes. CAR-transduced T cells were stained by anti-EGFR antibody to detect transduction efficiency of CAR on the day of harvest via flow cytometry. NTD represents the non-transduced cells as negative controls.

As per the research requirements, the transduced cells could be subjected to sorting before engaging in expansion and following functional assays, ensuring a high purity of CAR-expressing cells within the population. The transduced cells were subsequently expanded using recombinant human cytokines to support their growth and survival. For CAR-T cells, a combination of IL-7 and IL-15 was used, as these cytokines are known to promote T cell proliferation and survival (Zhou et al., 2019). Similarly, for CAR-MAIT cells, IL-12 and IL-18 were employed, as these cytokines have been shown to enhance the expansion and effector functions of MAIT cells (Ussher et al., 2014). Over a 10-to-12-day period, the transduced cells were cultured in the presence of these cytokines to facilitate their expansion to a satisfactory level for the subsequent assays. As demonstrated by the representative graph, CAR-T cells achieved over 70% CAR-positive cells, indicating successful transduction and sustained expression of the CAR construct. CAR-MAIT cells constituted approximately 55% of the cell population after expansion, further confirming the feasibility of producing CAR-MAIT cells (Figure 3.11). These results underscore the effectiveness of the transduction protocols employed in the study, laying the foundation for subsequent functional assays to evaluate the cytotoxic activity of the CAR-MAIT cells.

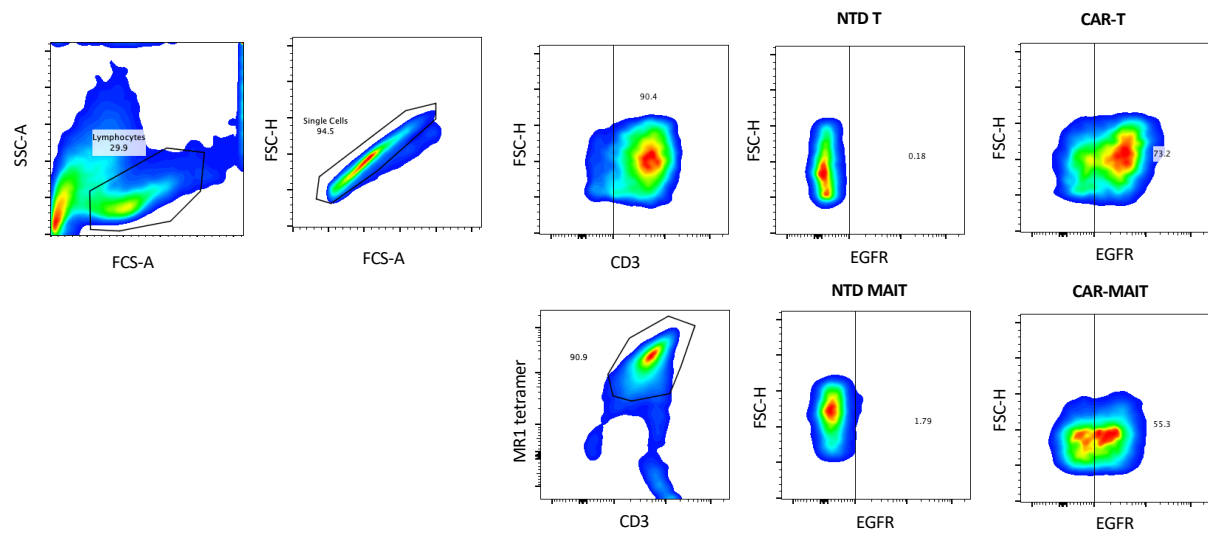


Figure 3.11. Generation of 3rd generation CD19-specific T cells and MAIT cells.

CAR-transduced T and MAIT cells were stained by anti-EGFR antibody to detect transduction efficiency after 10 days of culture. Most cells were CD3⁺ cells while the sorted MAIT cells also stayed in over 90% purity. NTD represents the non-transduced control cells as negative controls.

3.5. Discussion

In the pursuit of advancing CAR-based therapy, a third-generation anti-CD19 CAR was intricately designed. Two viral transduction systems have been established, with a parallel focus on optimizing the viral transduction protocols for embedding the CAR onto both conventional T cells and the distinctive MAIT cell subset for the subsequent functional evaluations. The strategic selection of anti-CD19, specifically the FMC63 monoclonal antibody, as the target epitope for CAR design, was predicated upon its proven specificity and high binding affinity to CD19, thereby establishing a solid and reliable foundation for evaluating the functional efficacy of MAIT cells in targeting B cell-related malignancies. The third-generation CAR-T constructs, featuring two co-stimulatory domains, have undergone functional and safety analysis in both preclinical and clinical investigations. By conducting *in vitro* testing of various CAR designs, it was determined that the construct incorporating both CD28 and 4-1BB signaling domains exhibited superior proliferation capacity and cytokine release (Zhao et al., 2015; Weinkove et al., 2019). Therefore, to enhance persistence and activation signaling, a design incorporating the CD28 and 4-1BB co-stimulatory molecules was devised. In the manufacturing process, the establishment of a reliable method for monitoring CAR-T cells is imperative. Traditionally, flow cytometry has been employed for the identification of cells carrying CARs. This involves utilizing antibodies targeting the extracellular regions of the molecule, such as the hinge via an anti-IgG Fc antibody or F(ab')₂ fragment. A more recent approach proposes the utilization of protein L derived from *Peptostreptococcus* for detecting CAR expression via flow cytometry (Zheng, Chinnasamy & Morgan, 2012; De Oliveira et al., 2013). However, both approaches exhibit cross-reactivity with IgG-like proteins, leading to non-specific staining. While there are specific reagents

designed for the detection of CAR-T cells, such as the biotinylated CD19 antigen-based fusion protein kit from Miltenyi Biotech, these reagents are expected to exhibit a low background staining due to their antigen specificity. However, they come with a high cost and are only suitable for a particular scFv (Schanda et al., 2021). In this context, tEGFR has been incorporated in the CAR design as an alternative marker for CAR detection. Importantly, anti-EGFR staining demonstrated minimal background and could be employed concurrently with other T cell antibodies during analysis. The robust correlation observed between anti-mouse IgG F(ab)₂ and anti-EGFR antibody staining further confirms the feasibility of its application. Beyond enabling the tracking of CAR-expressing cells, the expression of EGFR provides a supplementary safety mechanism. The presence of EGFR also allows for the elimination of CAR-expressing cells via EGFR-specific antibody Cetuximab in necessary circumstances, thereby fortifying the safety profile of the therapeutic approach (Kao et al., 2019).

Selecting an optimal gene delivery system is pivotal for successful CAR engineering. The utilization of virus-based gene delivery is prevalent in CAR-T cell manufacturing. The retrovirus platform, characterized by the establishment of a stable virus packaging cell bank, enables the consistent and stable production of retroviral vectors for CAR-T cell transduction within a short timeframe. On the other hand, the lentivirus platform is known for its adaptability and ability to transduce both dividing and non-dividing cells (Poorebrahim et al., 2019). Despite the transient nature of lentivirus production from 293T cells, it outperformed the retrovirus system by generating a higher virus titer. This characteristic enables the transduction of more cells with smaller amounts of virus stock, optimizing efficiency. However, unlike the stable retrovirus-producing cell line, 293T cells must be repeatedly transfected to produce lentivirus. The establishment of both virus transduction systems represents a

methodological endeavour to identify the optimal strategy for CAR transduction under various production conditions and manufacture needs. Further works could focus on establishing a lentivirus stable producer cell line to meet the demands of large-scale, cost-effective, high-titer production.

Given the low prevalence of MAIT cells within the circulating T cell pool, a prudent decision was undertaken to employ a purification method for the initial transduction tests of MAIT cells. Cell sorting employing PE-conjugated MR1 tetramer facilitated the isolation of a remarkably pure MAIT cell population for transduction. This strategic approach ensured the precision of the initial transduction assessment, mitigating potential interference from non-MAIT cells. The decision to utilize IL-12 and IL-18 as more specific cytokines for MAIT cell growth, as opposed to the generic IL-2, was motivated by the intention to prevent the potential overgrowth of non-MAIT cells within the MAIT cell population. Following activation with CD3/CD28 Dynabeads, both MAIT cells and conventional CD8⁺ T cells exhibited a similar clustering pattern. Measurement of activation markers revealed a robust upregulation of CD25 and CD69 in both MAIT cells and CD8⁺ T cells. However, MAIT cells demonstrated a lower level of expression in CD38 and HLA-DR compared to T cells. This disparity may indicate a slightly different activation pattern and downstream signaling triggered in these two distinct cell populations, suggesting potential subtleties in their responses to activation signals. Clinical reports suggest that higher CD38/HLA-DR co-expression in cytotoxic T cells was associated with superior elimination of malignant cells, yet it might be preferable for MAIT cells to demonstrate low CD38 and HLA-DR levels, as exhausted MAIT cells with compromised effector functions in tumour patients exhibited upregulated expression of HLA-DR, CD38 and IL-17A compared to the healthy samples (Henry et al., 2018; Shi et al., 2023). Further

investigations could shed light on these differences, contributing to a comprehensive understanding of MAIT cell activation mechanisms and their distinctive properties in comparison to conventional T cells. The successful viral transduction of CAR into MAIT cells represents a pivotal milestone, affirming the feasibility and effectiveness of extending CAR-T cell technology to the unconventional T cell subset, MAIT cells, via the established viral system. The strategic engineering and redirection of MAIT cell specificity hold substantial promise for diversifying and enhancing the therapeutic landscape of CAR-T cell applications across various malignancies.

In the upcoming chapter, the CAR-modified cells developed in this section were used to conduct *in vitro* experiments aimed at assessing the therapeutic potential of CD19-specific CAR-MAIT cells.

Chapter 4. Evaluating the functions of CD19-specific

CAR-MAIT cells

MAIT cells, characterized by their unique ability to rapidly respond to microbial antigens in the MR1-restricted manner and exhibit tissue-homing properties, have attracted increasing interest in recent years due to their potential therapeutic applications in cancer immunotherapy (Lukasik, Elewaut & Venken, 2020). In this chapter, MAIT cells were subjected to phenotypic characterization, and the transduced CAR-MAIT cells made from the previous chapter underwent functional evaluations to elucidate their efficacy in targeting and eliminating specific target cells *in vitro* and their therapeutic potential in allogeneic setting. By expanding the understanding of MAIT cell biology and their interaction with cancer cells and allogeneic cells, more ideas and evidence can be generated to determine the feasibility of MAIT cells as an alternative cell type for CAR therapy.

4.1. Phenotypical characteristics of MAIT cells

The distinctive surface markers exhibited by MAIT cells render them promising candidates for CAR-based therapy. In the analysis of T cell subsets, the examination of CD4 and CD8 expression reveals the immunophenotypic profiles that contribute to the understanding of cellular heterogeneity. For conventional CD3 T cells, the distribution between CD4 and CD8 subsets adheres to the well-established paradigm of helper (CD4⁺) and cytotoxic (CD8⁺) lineages, reflective of their distinct functional roles within the immune system. The distribution pattern of CD4 and CD8 in CD3 T cells typically adheres to a balanced ratio, a hallmark of a functional and responsive adaptive immune system. A substantial majority

(>85%) of MAIT cells predominantly exhibited a CD8⁺ phenotype, highlighting their cytotoxic potential. Merely a minority (<10%) of MAIT cells lacked both CD4 and CD8 markers, and approximately 1% were CD4⁺ (Figure 4.1). The prevalence of CD8⁺ MAIT cells aligns with the desired cytotoxicity associated with CAR-T cell therapy. Furthermore, MAIT cells exhibited a distinct tissue-homing profile, expressing chemokine receptors CCR5 and CCR6 (Figure 4.2). This characteristic is significant for CAR therapy as it implies that engineered MAIT cells may effectively target and infiltrate tissues, contributing to enhanced therapeutic outcomes. The expression of CCR5 and CCR6 suggests a propensity for MAIT cells to migrate to specific anatomical locations where their effector functions can be maximized. Memory effector markers on the circulating MAIT cells further enhance their potential for CAR-based therapy. The expression profile included CD45RO⁺, CD45RA⁻, CD62L⁻, and CCR7⁻ (Figure 4.3). CD45RO is associated with memory T cells, indicating that MAIT cells possess a memory phenotype. The absence of CD45RA, CD62L, and CCR7 suggests that MAIT cells are biased toward an effector memory state, emphasizing their readiness to mount rapid immune responses upon encountering antigens.

In summary, the collective expression of these surface markers not only highlights the distinctive identity of MAIT cells but also positions them strategically for effective CAR manufacturing. CD8 expression suggests their cytotoxic capabilities, while the tissue-homing markers CCR5 and CCR6 indicate their capacity to migrate to specific tissues. The presence of memory effector markers enhances the adaptability and responsiveness of MAIT cells, as key attributes for effective CAR-T cell therapy. These findings solidify our understanding of the unique surface profile of MAIT cells.

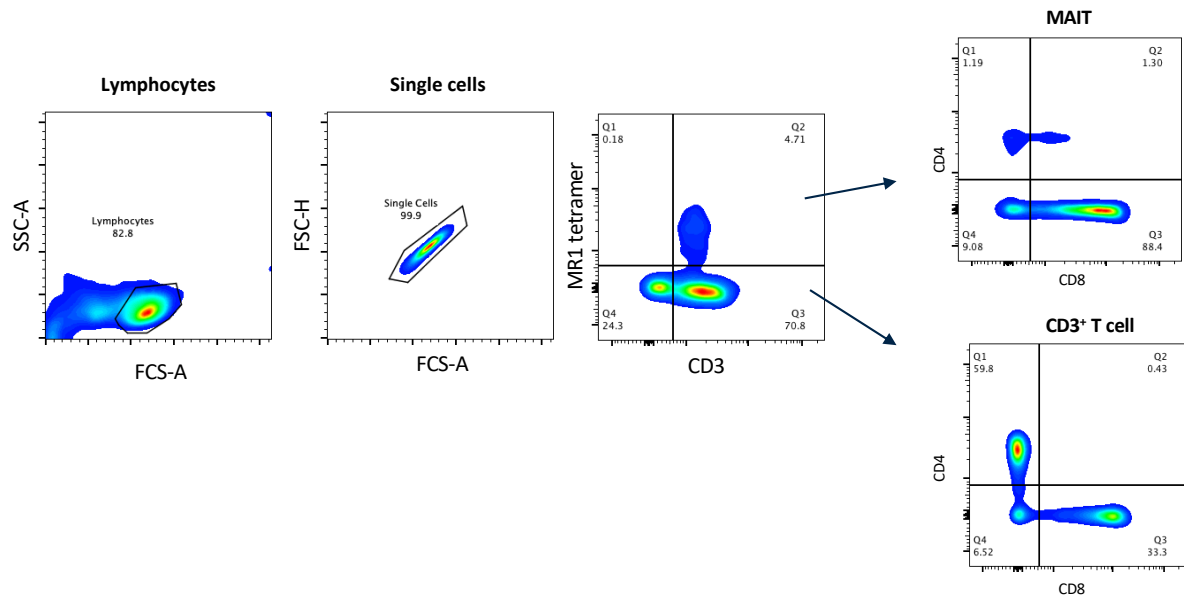


Figure 4.1. Surface phenotyping of MAIT cells showing CD4 and CD8 distribution.

Representative flow graph depicting CD4 and CD8 distribution within conventional CD3⁺ T cells and MAIT cells. Conventional T cells were identified as CD3⁺, while MAIT cells were defined as CD3⁺ MR1 tetramer⁺ cells. The staining for CD4 and CD8 was conducted to assess the frequency of each population.

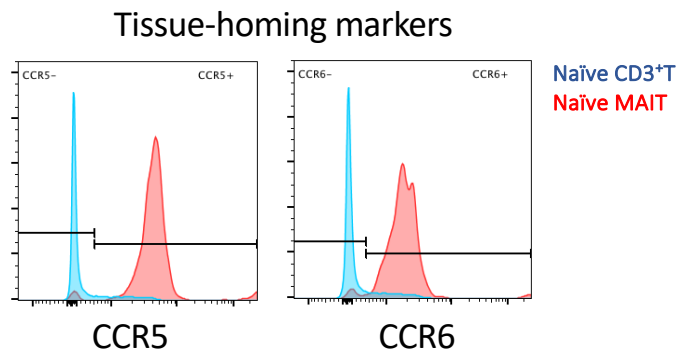


Figure 4.2. Surface phenotyping of MAIT cells showing expression of tissue-homing markers.

Representative histogram overlays of expression of tissue-homing markers, CCR5 and CCR6, on MAIT cells (red histograms), non-MAIT CD3⁺ T cells (blue histograms).

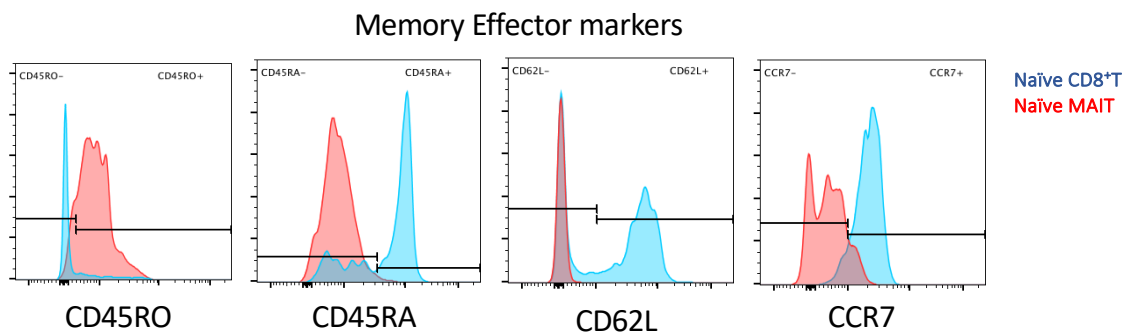


Figure 4.3. Surface phenotyping of MAIT cells showing memory effector markers.

Representative histogram overlays of expression of memory effector markers, CD45RO, CD45RA, CD62L and CCR7, on MAIT cells (red histograms), non-MAIT CD8⁺ T cells (blue histograms).

4.2. Targeting CD19⁺ B cell lines using CAR-MAIT cells

After validating CAR expression in T cells and MAIT cells in Chapter 3, their *in vitro* anti-tumour efficacy was assessed using the CD19⁺ B lymphoblastoid cell line (BLCL). The BLCLs were engineered by infecting B lymphocytes with the Epstein-Barr virus (EBV), thereby transforming them into immortalized EBV-bearing BLCLs. The flow analysis confirmed the expression of CD19 on the surface of BLCLs (Figure 4.4 A). To evaluate the cytotoxic potential of the CAR-expressing effector MAIT cells, a non-radioactive killing assay was conducted. Both anti-CD19 CAR-T and CAR-MAIT cells were co-cultured with CFSE-labelled target BLCLs at different effector-to-target ratios (1:1 and 5:1) for a duration of 20 hours. Subsequently, dead cells were stained with DAPI, and the surviving target BLCL cells were quantified using Countbright beads and flow analysis (Figure 4.4 B). As a control, non-transduced T and MAIT cells were used as the background ablation. The results demonstrated a remarkable cytotoxic capability of both anti-CD19 CAR-T and CAR-MAIT cells, achieving a 70-80% reduction in BLCLs at a 5:1 effector-to-target cell ratio (Figure 4.4 C). These results not only underline the specificity of CAR-MAIT cells in killing target cells but also highlight their ability to exert a comparable cytotoxic impact as conventional CAR-T cells. The findings propel the notion that MAIT cells, when engineered with a CAR construct, can indeed serve as a novel and potent cell type for CAR-based immunotherapy. This observation opens avenues for the integration of unconventional T cell subset, MAIT cells, into the expanding landscape of CAR-T cell technology, offering innovative approaches in the treatment of malignancies.

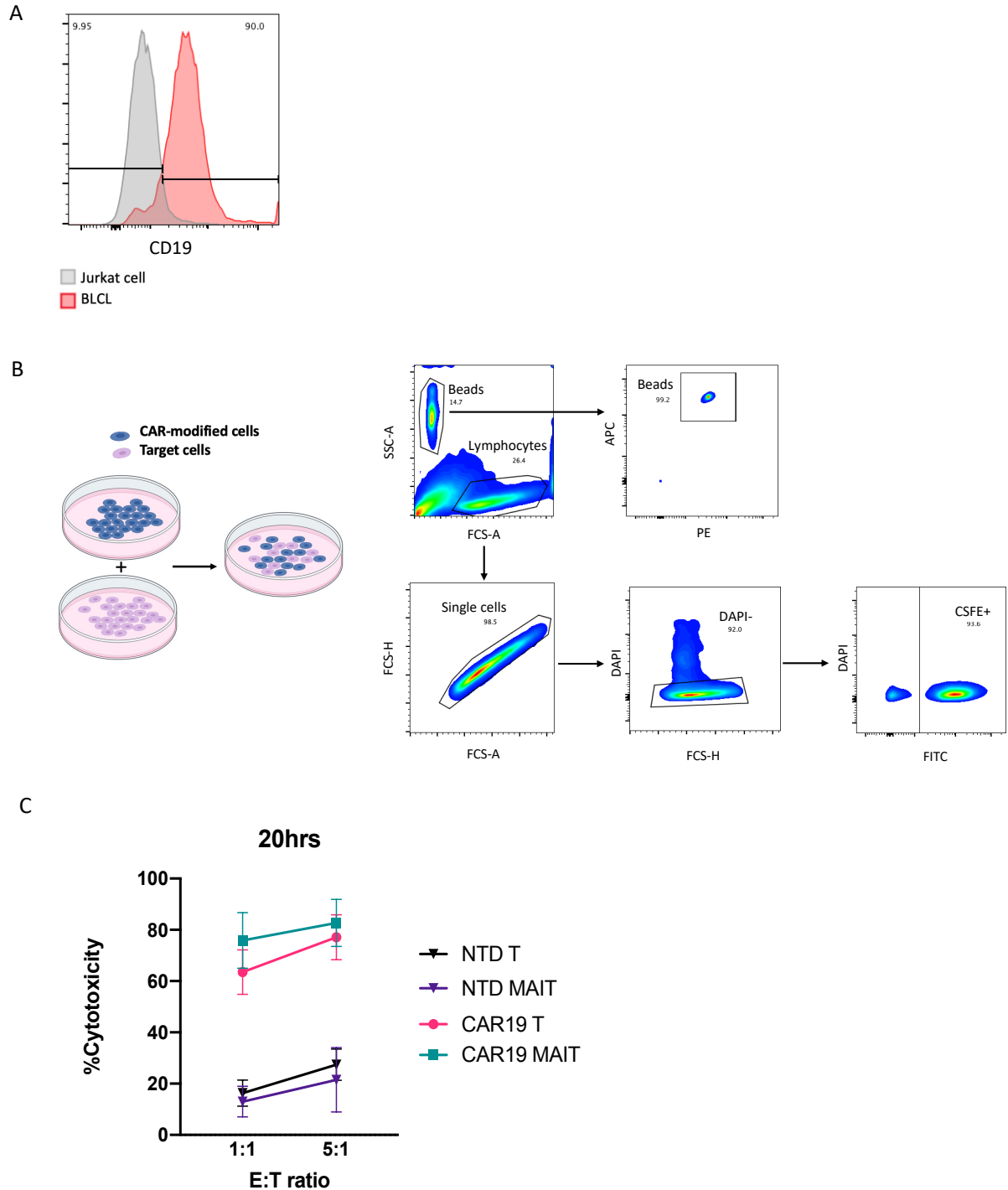


Figure 4.4. Killing of anti-CD19 CAR-T and CAR-MAIT cells against BLCL.

(A) B lymphoblastoid cell (red histogram) was stained by the anti-CD19 antibody to assess and validate the CD19 expression level on the cell surface by flow cytometry analysis. T cell line, Jurkat cell (grey histogram), was used as negative control. (B) Gating strategy for flow analysis of cytotoxicity. CFSE-labelled B cells were mixed with effector CAR-modified cells. After co-culturing, cells were collected and stained by DAPI to distinguish live and dead cells. Target cells were identified with CFSE staining. A fixed volume of Countbright beads were added to each sample. The samples were loaded to flow cytometry for absolute quantification. The number of surviving

BCL was calculated as: cells in tube = (cells collected/ beads collected) x total beads added to the tube. (C) Percent cytotoxicity of anti-CD19 CAR-T (CAR19 T) and CAR-MAIT cells (CAR19 MAIT) targeting BLCL. CAR-expressing effector cells were co-cultured with BLCL at the ratios of 1:1 and 5:1 respectively for 20 hours. NTD represents non-transduced T or MAIT cells as negative controls. Data with error bars are represented as mean $\pm$ SEM. Data from a single experiment; results are representative of two independent biological replicate experiments.

4.3. Alloreactive Potential of MAIT Cells

In addition to evaluating the killing capacity for specific target elimination, assessing the alloreactive potential is crucial in judging the suitability of CAR-MAIT cells for cellular therapy, particularly in the context of allogeneic immunotherapy. A comprehensive mixed lymphocyte reaction (MLR) assay was conducted to compare the alloreactive responses of MAIT cells with conventional T cells. CFSE-labelled human PBMCs from one donor were cultured with allogeneic PBMCs from another donor at a 1:1 ratio for 6 days, providing a robust model to evaluate the proliferative capacity of both cell types. DAPI was used to distinguish live and dead cells. MAIT cells were identified among conventional CD3⁺ T cells through staining with the MR1 tetramer (Figure 4.5 A). The results of the CFSE staining revealed distinct patterns in the proliferative response of non-MAIT T cells and MAIT cells when exposed to allogeneic cells. MAIT cells exhibited a significantly lower level of proliferation compared to conventional T cells in this allogeneic setting. The quantification of CFSE dilution at the end of the culture period demonstrated a subdued proliferative activity in MAIT cells, suggesting a limited alloreactive response (Figure 4.5 B). To validate these findings, appropriate controls were included in the experimental design. The negative control, involving a culture with autologous PBMCs, served as a baseline for assessing the intrinsic proliferation of cells without the influence of alloreactivity. The positive control, utilizing CD3 beads to induce a strong proliferation response, ensured the sensitivity of the experimental system to detect alloreactive potential (Figure 4.5 B). Results from multiple recipient/donor pairs consistently demonstrated that MAIT cells exhibited minimal proliferation in response to allogeneic stimulation, highlighting a significantly lower alloreactive potential compared to conventional T cells (Figure 4.6).

To sum up, the analysis of alloreactive potential adds valuable insights to our understanding of MAIT cell behaviour in the context of allogeneic interactions and has profound implications for the potential use of MAIT cells in allogeneic cellular therapy. The reduced alloreactive response suggests a favourable safety profile, minimizing the risk of graft-versus-host reactions often associated with conventional T cells in allogeneic settings. This characteristic makes MAIT cells an attractive candidate for allogeneic CAR-based therapies, providing a foundation for the development of safer and more effective immunotherapeutic strategies.

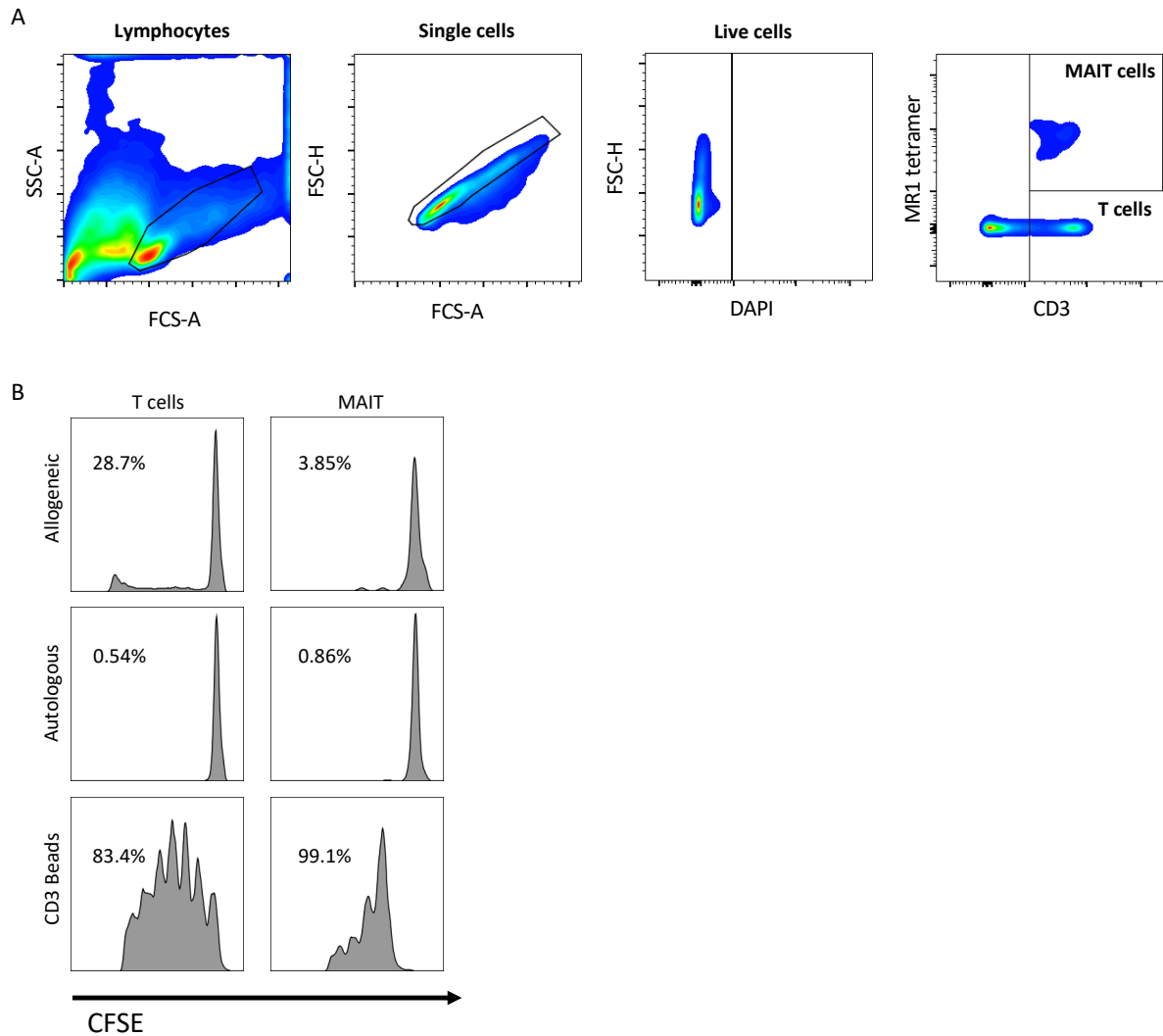


Figure 4.5. Alloreactive response of T cells and MAIT cells *in vitro*.

(A) Gating strategy for flow analysis of alloreactive response to allogeneic stimuli. Cells were stained by DAPI to distinguish live and dead cells. Conventional T cells were identified as CD3⁺, while MAIT cells were defined as CD3⁺ MR1 tetramer⁺ cells. (B) Representative CFSE staining of T cells and MAIT cells in response to allogeneic cells *in vitro*. CFSE-labelled human PBMCs (10⁶/mL) were co-cultured in the presence of allogeneic (upper panel) or autologous (middle panel) PBMCs at a 1:1 ratio for 6 days. A culture with CD3 beads (lower panel) to was used as a positive control to assess the proliferation response. Proliferation of both non-MAIT T cells and MAIT cells was quantified by CFSE dilution at the end of the culture period. Each percentage value in the histogram represents the proportion of cells that have undergone proliferation in response to the allogeneic stimuli.

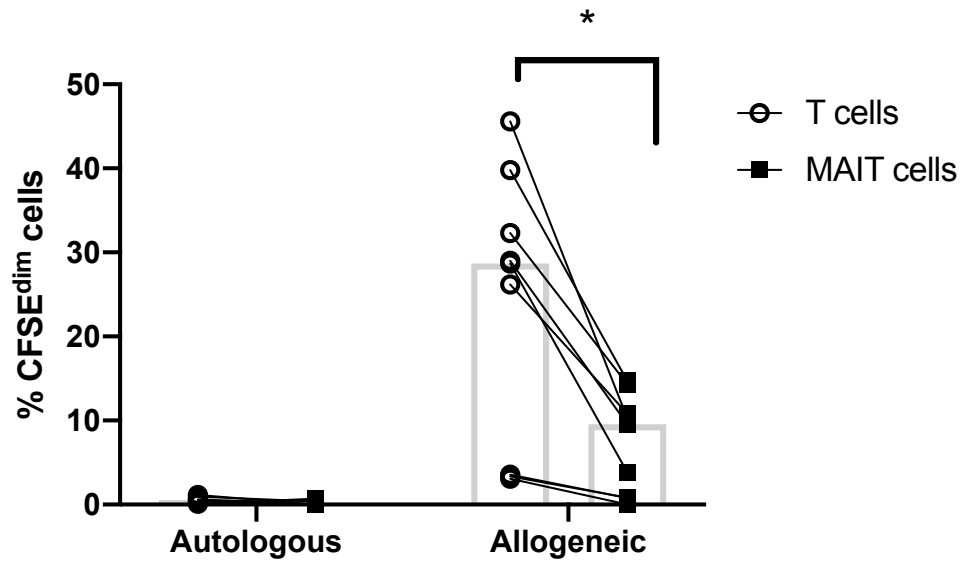


Figure 4.6. Percentage of alloreactive T cells and MAIT cells in response to *in vitro* PBMC stimulation.

CFSE-labelled human PBMCs ($10^6/\text{mL}$) were co-cultured with allogeneic or autologous PBMCs for 6 days to assess the proliferation of alloreactive cells. The percentage of proliferating responder cells (CFSE^{dim}) within each population was evaluated. Data from two independent experiments ($n=2$) with a total of nine recipient/donor pairs were included in this experiment. T cells are represented by circles, while MAIT cells are represented by squares. Connecting lines between T cells and MAIT cells from the same recipient/donor pair illustrate the disparity in their alloreactive responses. A two-tailed unpaired Student's t-test was used for significance analysis.

* = $p < 0.05$.

4.4. Discussion

Motivated by the growing interest in expanding the repertoire of CAR-based therapies, our exploration into the unique attributes of MAIT cells aims to explore their potential in therapeutic applications. The distinctive features of MAIT cells, ranging from their surface marker profile to their functional responses, present a compelling avenue for innovation in cellular therapy.

The distinctive markers expressed on MAIT cells play a crucial role in shaping their functionality. MAIT cells exhibit a predominant CD8⁺ phenotype, with a minority characterized as double-negative (CD4⁻CD8⁻), and a very small fraction expressing CD4, setting them apart from conventional T cells. The predominant CD8⁺ nature of MAIT cells aligns with findings from various studies (Brozova, Karlova & Novak, 2016; Gherardin et al., 2018a; Koay et al., 2019). There are no apparent phenotypical or functional distinctions between the two primary subpopulations, CD8⁺ and double-negative MAIT cells. Among these, the CD8⁺ subset generally exhibits a higher prevalence of pro-inflammatory and cytotoxic cytokines, such as TNF- α , IFN- γ , and granzyme B, compared to the double-negative MAIT cells (Brozova, Karlova & Novak, 2016). The prevalence of CD8⁺ MAIT cells aligns strategically with the cytotoxicity desired in CAR-T cell therapy. In other words, the inherent cytotoxic potential of CD8⁺ MAIT cells positions them as promising candidates for CAR-based immunotherapy, underscoring their suitability for targeted cancer cell elimination. On the other hand, CD4⁺ MAIT cells, despite their limited representation, exhibit distinctive features compared to the other MAIT subpopulations in terms of antigen expression and cytokine production. A study indicates that CD4⁺ MAIT cells differ in the expression of certain markers such as CXCR6, CD56, NKG2D, and

NKG2A, and demonstrate an increased production of IL-2, a cytokine with pivotal roles in immune regulation and coordination (Gherardin et al., 2018a). The functional implications of these unique characteristics remain to be fully elucidated. Currently, given the relatively low abundance of CD4⁺ MAIT cells within the MAIT cell population, deliberate isolation of each MAIT cell subpopulation during CAR manufacturing has not been pursued. This decision is primarily influenced by considerations of practicality and efficiency, given the challenges associated with isolating rare subsets. However, future research endeavours should delve deeper into unravelling the precise roles and functionalities of CD4⁺ MAIT cells within the context of CAR-T cell therapy. These investigations hold the promise of yielding valuable insights into the contributions of CD4⁺ MAIT cells and their potential impact on the outcomes of CAR-T cell-based treatments.

The expression of tissue-homing markers, CCR5 and CCR6, on MAIT cells is noteworthy, suggesting an inherent capacity for migration to specific tissues. CCR5 and CCR6 are critical for T cell homing to inflamed tissues, suggesting that CAR-MAIT cells, by expressing these markers, might exhibit enhanced cell trafficking and targeting of tumour sites or other diseased tissues. Additionally, the memory effector phenotype of MAIT cells, characterized by the CD45RO⁺, CD45RA⁻, CD62L⁻, and CCR7⁻ profile, aligns with the memory features crucial for persistent and rapid responses against encountered antigens. This finding is consistent with the recent MAIT cell phenotypic analysis (Gherardin et al., 2018a). The signature of memory effector markers implies the ability to mount a rapid and potent response upon antigen recognition, reinforcing the notion that CAR-MAIT cells possess robust effector functions crucial for targeted immune responses.

The exceptional killing efficacy of CAR-MAIT cells, demonstrated through the targeted elimination of CD19⁺ B lymphoblastoid cells, is a significant step in validating their therapeutic potential. Achieving a comparable level of cytotoxicity as conventional CAR-T cells, CAR-MAIT cells exhibited a remarkable ability to induce cell death in the target population. This outcome substantiates the idea that MAIT cells, when engineered with a CAR construct, can serve as an effective cytotoxic force, expanding the repertoire of cellular therapy targets beyond conventional T cells. The robust killing efficacy exhibited by CAR-MAIT cells, as evidenced in this study, resonates with results reported in a recent publication. In that study, human MAIT cells were engineered with CD19-specific and Her2-specific CAR constructs. Their outcomes further underscored the potential of CAR-MAIT cells in cancer immunotherapy by efficiently and selectively targeting respective cancer cells, including B cell lymphomas and a Her2⁺ MDA-231 solid tumour cell line. Intriguingly, MAIT cells exhibited high cytotoxicity, effectively eliminating target cells even at an E:T ratio of 1:32 (Dogan et al., 2022).

The investigation into the alloreactive potential of MAIT cells yielded encouraging results. Unlike conventional T cells, MAIT cells exhibited a markedly lower proliferative response to allogeneic stimulation, indicating limited alloreactivity. This finding holds significance, particularly in the context of allogeneic immunotherapy, where mitigating graft-versus-host reactions is crucial. The diminished alloreactive response positions CAR-MAIT cells as a potentially safe and effective option for allogeneic cellular therapies, broadening their clinical applicability. These findings are in line with results reported in a recent publication, proving that human MAIT cells lack alloreactive potential. *In vivo* model using the immunodeficient mice with simulating T-cell-mediated xenogeneic graft-versus-host disease demonstrated that human MAIT cells did not undergo significant expansion or accumulation in tissues.

Signals from allogeneic cells, combined with cytokine exposure, failed to induce sustained proliferation of MAIT cells (Tourret et al., 2021). Therefore, both *in vitro* and *in vivo* results confirmed that MAIT cells do not undergo hyperactivation typically associated with alloreactivity. In other words, MAIT cells may overcome HLA disparity barriers and minimize adverse effects in adoptive immune therapy, ensuring safety in allogeneic settings.

In summary, the development of CAR-MAIT cells holds great promise as a novel avenue to address the challenges associated with personalized CAR-T cell manufacturing. The distinct phenotypic and functional characteristics of MAIT cells, including their prevalent CD8⁺ phenotype, tissue-homing markers (CCR5 and CCR6), and memory effector markers (CD45RO⁺CD45RA⁻CD62L⁻CCR7⁻), align well with the requirements for effective CAR-T cell therapy. The robust cytotoxic potential of CAR-MAIT cells further supports their suitability for CAR-based applications. Importantly, the findings suggest that CAR-MAIT cells could offer a safe and efficacious platform, minimizing the risk of adverse effects associated with alloreactivity. The low alloreactive potential observed in MAIT cells implies a reduced likelihood of GVHD, positioning CAR-MAIT cells as a potential universal cell source for allogeneic adoptive immune therapy. In this context, CAR-MAIT cells could be developed as 'off-the-shelf' CAR products, providing a readily available and standardized treatment option for a broader patient population. This approach could streamline the production process, reduce costs, and increase accessibility to CAR-T cell therapies, making them more widely applicable in clinical settings. Since the concept of CAR-MAIT cells has been validated, in the subsequent experimental chapter of this thesis, diverse methodologies for *in vitro* expansion of MAIT cells were investigated to ascertain the optimal approach for clinical-scale manufacturing.

Chapter 5. Development of MAIT cell-specific *in vitro* expansion strategies

Having validated the concept of generating CAR-MAIT cells and assessing their therapeutic potential in the previous chapters, the establishment of a standard protocol for CAR-MAIT manufacturing becomes principal. Challenges inherent in developing CAR-MAIT cell therapy revolve around the complexities of expanding cells to therapeutic levels and standardizing the production protocol. Given the relatively small proportion of MAIT cells within the total circulating T cell population, a primary objective is to develop optimal MAIT cell-specific expansion assays conducive to large-scale CAR-MAIT cell production. Although the application of CD3/CD28 Dynabeads to stimulate MAIT cells has demonstrated success in the previous section, there are inherent challenges associated with this approach. To utilize the CD3-based stimulation approach effectively, MAIT cells must be carefully isolated and purified to prevent the overgrowth of non-MAIT cells. Given that MAIT cells constitute only a small fraction of the circulating T cell population, achieving a highly pure population demands a substantial amount of initial donor blood for isolation. This necessity poses a practical challenge for cost-effective and scalable manufacturing, especially in the context of large-scale therapeutic production. Recognizing these limitations, the exploration of a MAIT cell-specific expansion strategy becomes imperative. This strategic shift aligns with the paradigm seen in the manufacturing processes of the other unconventional T cell subsets, such as $\gamma\delta$ T cells for CAR therapy, where activation with Zoledronate on day 0 resulted in the selective proliferation of $\gamma\delta$ T cells (Rozenbaum et al., 2020). By employing a MAIT cell-specific expansion strategy, the aim is to optimize the manufacturing process, enabling the generation

of CAR-MAIT cells in a more scalable and economically viable manner. This approach holds promise not only for overcoming logistical constraints associated with isolating rare cell populations but also for advancing the feasibility of implementing CAR-MAIT cell therapy in diverse clinical contexts. Therefore, this chapter delves into the exploration and refinement of a MAIT cell-specific expansion protocol to address these challenges.

5.1. MAIT cell-specific expansion strategy with anti-V α 7.2 antibody

Using antibodies to stimulate T cells is a strategy commonly employed in immunotherapy research and clinical applications. Antibodies offer a targeted approach to specifically activate and expand T cell populations *ex vivo* by precisely targeting the surface molecules expressed on T cells, such as the CD3 complex, which is essential for TCR signaling and activation (Abraham et al., 2008; Ghaffari et al., 2021). By engaging CD3 on the T cell surface, antibodies trigger downstream signaling cascades that mimic physiological T cell activation, leading to proliferation, cytokine production, and effector function (Ghaffari et al., 2021). In addition to anti-CD3 antibodies (OKT3), antibodies targeting the antigens expressed on unconventional T cell subsets, such as $\gamma\delta$ T cells, have been utilized for stimulation and expansion. The anti-TCR $\gamma\delta$ antibody offers a selective method to activate and expand $\gamma\delta$ T cells within PBMCs, resulting in a substantial increase in $\gamma\delta$ T-cell percentages. This expansion level is comparable to that achieved using phosphoantigen stimulation, demonstrating the efficacy of antibody-based activation for $\gamma\delta$ T-cell enrichment (Kang et al., 2009; Zhou et al., 2012).

Building on the effectiveness of antibody-based stimulation for expanding T cells, a similar strategy was employed here to evaluate the effect on MAIT cell expansion. Since MAIT cells are characterized by the expression of the invariant TCR α chain V α 7.2-J α 33, the anti-V α 7.2

antibodies from different clones were used in this study to specifically target and stimulate MAIT cells, leveraging their ability to bind to the V α 7.2 domain of the cell receptor. 5 μ g/mL of anti-V α 7.2 antibodies, along with/without the functional grade anti-CD28 antibody (clone CD28.2), were coated on the plate prior to stimulation (Figure 5.1 A). Various clones of anti-V α 7.2 antibodies were used in this study, including REA179, 3C10, and OF5A12, in the presence of IL-2. Additionally, anti-CD3 antibody with/without the functional grade anti-CD28 antibody was utilized as a control to stimulate T cells. However, over the 6-day culture period, there was no significant change in the frequency of MAIT cells, irrespective of the anti-V α 7.2 antibody clones used or the addition of anti-CD28 antibody (Figure 5.1B). The minor increase in MAIT cell numbers observed upon stimulation with the anti-CD3 antibody was attributed to the concurrent expansion of non-MAIT T cells within the PBMC population. This proliferation of non-MAIT T cells could potentially outpace the expansion of MAIT cells, resulting in a relatively minimal fold change in the MAIT cell population. A series of concentrations of anti-V α 7.2 antibody (taking clone 3C10 as an example) were employed to assess any potential effects on MAIT cell expansion. However, no significant differences in the concentrations were observed regarding MAIT cell fold changes (Figure 5.1 C). This outcome suggests that the anti-V α 7.2 antibody may not effectively induce MAIT cell expansion under the current conditions.

Surprisingly, flow staining revealed that, apart from CD161⁺ MAIT cells, the anti-V α 7.2 antibody induced the expansion of V α 7.2⁺ CD161⁻ non-MAIT cells. Representative flow cytometry graphs illustrated that V α 7.2⁺ CD161⁻ cells increased from 1.31% to 25% over 6 days with anti-V α 7.2 antibody (3C10) in the presence of anti-CD28 antibody, IL-2, and IL-15. While MAIT cells (Figure 5.2 A). Their fold expansion reached 10 to 15, which was significantly

higher than that of MAIT cells. In contrast, MAIT cells exhibited a minor frequency change from 3.74% to 8.23%, resulting in approximately 2-to-3-fold expansion (Figure 5.2 A and B).

Taken together, the results obtained from the stimulation of MAIT cells with anti-V α 7.2 antibody reveal that anti-V α 7.2 antibody may not be effective in stimulating MAIT cell proliferation under the given conditions. However, the anti-V α 7.2 antibody (3C10) successfully induced robust proliferation of non-MAIT cells, particularly the V α 7.2⁺ CD161⁻ cells, while its impact on MAIT cells was comparatively limited. The unexpected expansion of V α 7.2⁺ CD161⁻ non-MAIT cells in response to anti-V α 7.2 antibody stimulation raises intriguing questions about the specificity and selectivity of this antibody. This discrepancy underscores the complexity of antibody-mediated stimulation and highlights the need for further investigation into the precise mechanisms governing the interaction between anti-V α 7.2 antibody and different T cell subsets. Additionally, exploring the potential functional differences between MAIT cells and V α 7.2⁺ non-MAIT cells could provide valuable insights into the specific signaling pathways and molecular processes involved. Due to the minimal efficacy observed with the antibody stimulation approach for MAIT cell-specific expansion, this research continued to explore the viability of alternative stimulation methods.

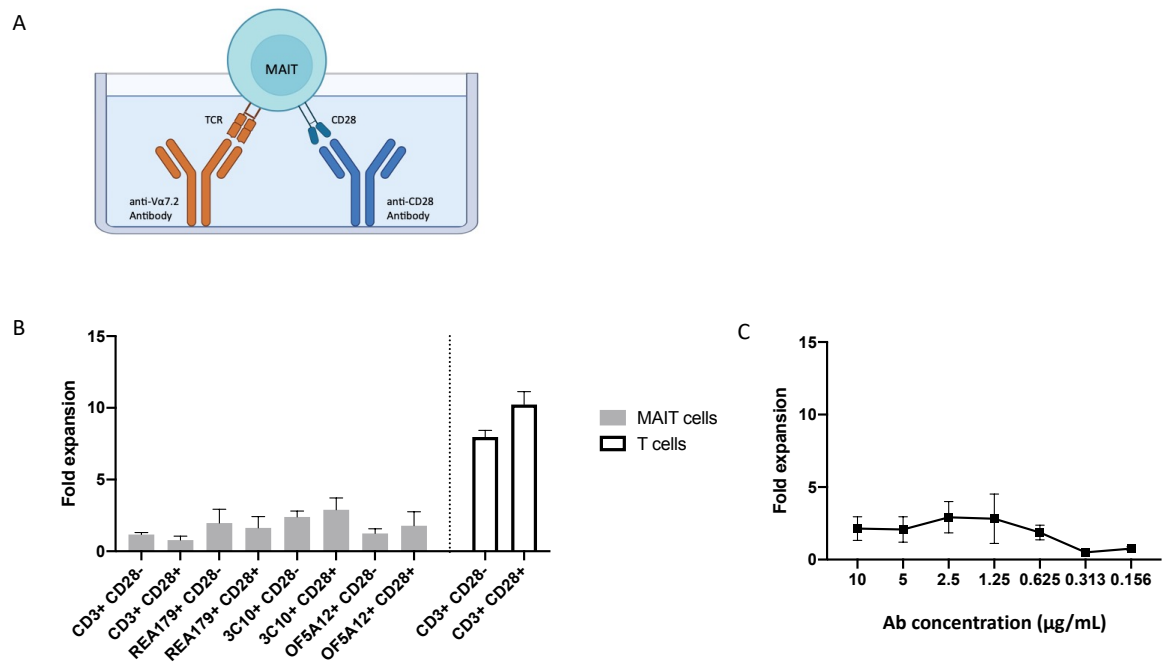


Figure 5.1. MAIT cells stimulation by anti-Vα7.2 antibodies.

(A) Schematic representation of the PBMC assay: MAIT cells were stimulated by coating the plate with anti-Vα7.2 antibodies and anti-CD28 antibodies. Figure modified using Biorender. (B) Fold expansion of T cells and MAIT cells within PBMCs over a 6-day culture period with plate-coated antibody stimulation. Anti-Vα7.2 antibodies (clones REA179, 3C10, and OF5A12) at a concentration of 5 μg/mL, with or without functional grade anti-CD28 antibody (clone CD28.2), were used for plate coating. Anti-CD3 antibody (clone OKT3), with/without anti-CD28 antibody were used as controls. MAIT cell frequency was assessed by flow cytometry on day 0 and day 6, and fold expansion was calculated by dividing the MAIT cell amount at the end of the culture by the initial amount. (C) Evaluation of the stimulatory effect of anti-Vα7.2 antibodies (3C10) on MAIT cells under various concentrations over 6 days (μg/mL). Data with error bars are represented as mean ± SEM. PBMCs from three different normal donors were used. Results are representative of two independent biological replicate experiments.

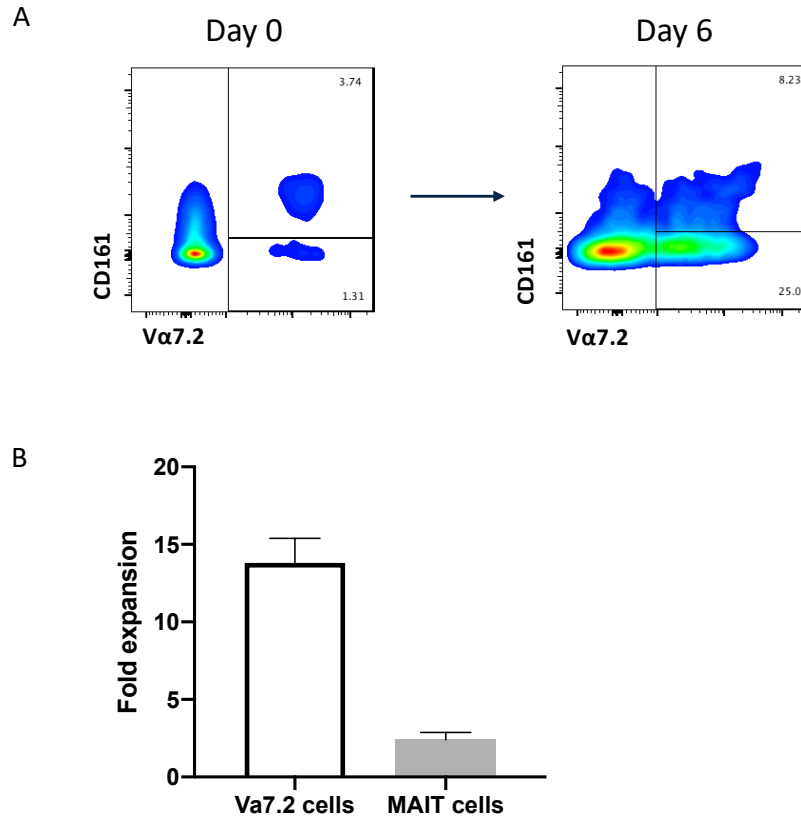


Figure 5.2. Non-MAIT Va7.2⁺ CD161⁻ cells expansion by anti-Va7.2 antibodies.

(A) Representative flow cytometry plots of changes in frequency of Va7.2⁺ CD161⁻ cells and MAIT cell in PBMCs over a 6-day period following stimulation with anti-Va7.2 antibody (3C10) in the presence of anti-CD28 antibody and IL-2/IL-15. (B) Fold expansion of Va7.2⁺ CD161⁻ cells and MAIT cells within PBMCs over a 6-day culture period with anti-Va7.2 antibody. Cell frequency was assessed by flow cytometry on day 0 and day 6, and fold expansion was calculated by dividing the cell amount at the end of the culture by the initial amount. Data with error bars are represented as mean ± SEM. PBMCs from five different normal donors were used.

5.2. MAIT cell-specific expansion strategy using 5-OP-RU

Unlike conventional T cells, MAIT cells do not respond to traditional antigenic stimulation through the TCR-MHC pathway. Instead, they recognize microbial metabolites presented by MR1. Specifically, 5-OP-RU, a riboflavin intermediate, serves as a potent ligand for the MR1 molecule. This metabolite binds to MR1 on APCs within PBMCs and is subsequently presented to MAIT cells (Figure 5.3 A). The interaction between 5-OP-RU and MR1, as perceived by the MAIT cell TCR, initiates the activation of MAIT cells (Hinks & Zhang, 2020; Hagel et al., 2020). Studies have demonstrated that direct injection of synthetic 5-OP-RU, in combination with other activation agents, induces MAIT cell expansion in mice to combat bacterial infection (Vorkas et al., 2020). Expanding upon these principles, the addition of the MR1 ligand 5-OP-RU directly into PBMCs presents an alternative approach to stimulate MAIT cells, wherein natural APCs could present 5-OP-RU antigens to MAIT cells. This strategy capitalizes on the intrinsic antigen-presenting capabilities of host APCs, potentially augmenting the efficiency and specificity of MAIT cell activation and expansion. In this study, the use of synthetic 5-OP-RU to selectively stimulate MAIT cells within PBMCs yielded compelling findings. MAIT cells exhibited successful activation in response to 5-OP-RU and underwent dose-dependent expansion over a 6-day period in the presence of IL-2. Measurement of the fold expansion of MAIT cells revealed a significant increase, with their population expanding approximately ten-fold within the PBMC population (Figure 5.3 B). For a representative donor with only 2.11% MAIT cells among circulating T cells, the addition of 100 nM 5-OP-RU resulted in a marked increase to 16.7%, with a further rise to 20.2% observed upon supplementation with 1000 nM 5-OP-RU (Figure 5.3 C). These findings underscore the efficacy of 5-OP-RU in selectively stimulating and expanding MAIT cells via the natural APCs within PBMCs.

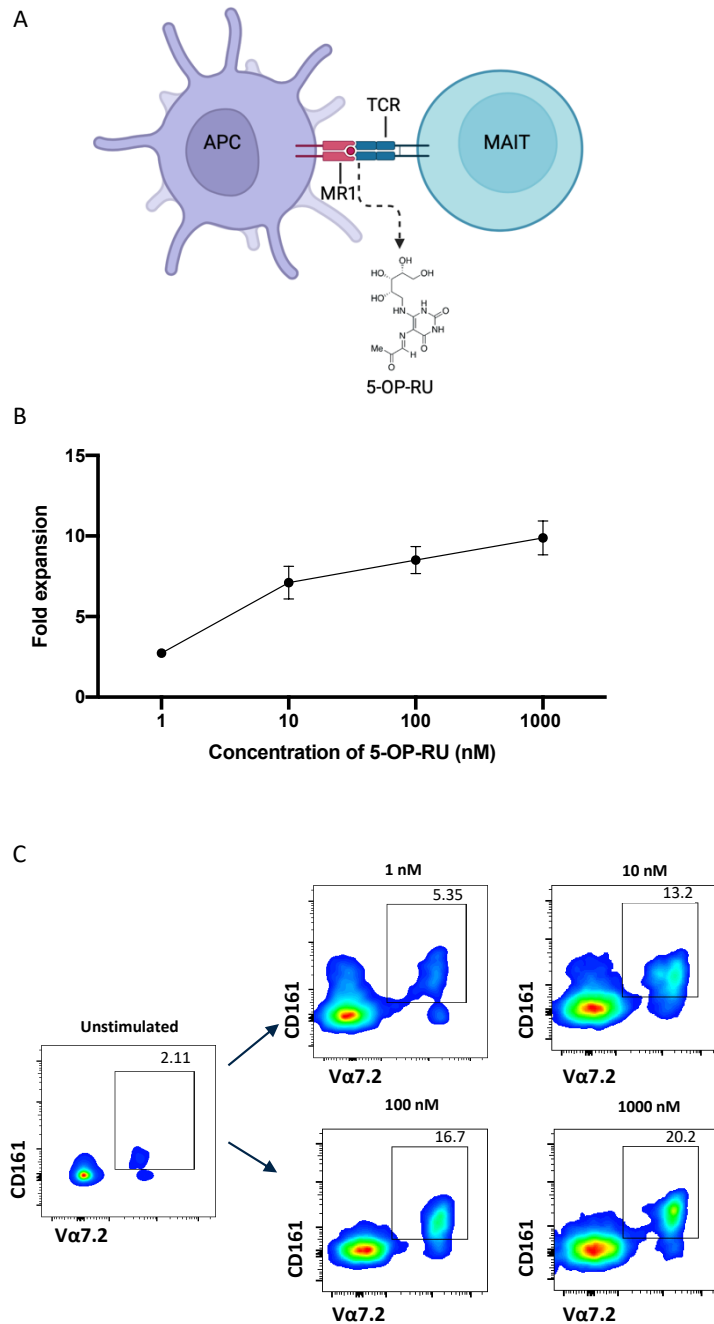


Figure 5.3. Dose-dependent expansion of MAIT cells with 5-OP-RU stimulation in the presence of APCs.

(A) Schematic graph of the PBMC assay: APCs within PBMCs presenting 5-OP-RU to MAIT cells. Figure modified using Biorender. (B) Fold expansion of MAIT cells within PBMCs over a 6-day culture period with the activation of 5-OP-RU. Different levels of 5-OP-RU were introduced into the PBMC culture, and the frequency of MAIT cells was evaluated using flow cytometry on both day 0 and day 6. The fold expansion was calculated by dividing the MAIT cell amount at the end of the culture by the initial amount. Data with error bars are represented as mean $\pm$ SEM. PBMCs from three different normal donors were used. (C) Representative flow cytometry plots of changes in MAIT cell frequency under various concentrations of 5-OP-RU.

5.3. MAIT cell-specific expansion strategy using artificial APC beads

The proven efficacy of 5-OP-RU in stimulating MAIT cells has prompted further exploration into the development of artificial APC beads to elicit a response from MAIT cells, mirroring the role of CD3 beads in stimulating T cells. This strategic shift towards MR1 beads is motivated by their potential advantages in the context of CAR-MAIT cell manufacturing. Unlike soluble compounds, these beads offer a tangible and controllable means of stimulation during manufacturing, ensuring a cleaner final cell product. Departing from dependence on natural APCs to present antigens to MAIT cells, the beads would serve as artificial APCs for antigen presentation, offering an alternative method for MAIT cell activation. The composition of these artificial APC beads involves streptavidin beads linked with biotinylated 5-OP-RU-loaded human MR1 molecules and anti-human CD28 monoclonal antibody (Figure 5.4 A). To generate the MR1 beads, the biotinylated 5-OP-RU loaded MR1 molecules and biotin-conjugated anti-human CD28 antibody were attached to the 2.8 μm cell-size magnetic Dynabeads, which are covalently linked to streptavidin. The MR1 molecules Different ratios of these components (CD28:MR1=0:4, 1:3, 2:2, 3:1, and 4:0 respectively) were mixed in a solution to determine the optimal proportion, followed by an overnight incubation and washing. The multiple tetrameric complexes on the MR1 beads mimic natural antigen presentation, while the addition of CD28 provides a co-stimulatory signal crucial for optimal MAIT cell activation. To validate the effective immobilization of MR1 tetramers and anti-CD28 on the beads, these prepared MR1 beads underwent staining with an anti-MR1 and anti-mouse IgG antibody, respectively. The flow cytometry analysis revealed efficient binding of both MR1 tetramers and anti-CD28 mAb to the surface of the streptavidin beads (Figure 5.4 B). In the conducted experiments, the impact of MR1 beads on MAIT cell expansion within

PBMCs was assessed. PBMC cultures were treated with beads bearing different ratios of CD28 and MR1 in the presence of IL-2. After 6 days of culture, the results revealed that MAIT cells within PBMCs exhibited robust and specific stimulation in response to the beads. Interestingly, the CD28:MR1 ratio of 0:4 and 1:3 demonstrated a particularly notable effect, resulting in a higher fold expansion of MAIT cells compared to other ratios (Figure 5.4 C). This suggests that a higher proportion of MR1 tetramer on the beads contributes to an enhanced proliferation of MAIT cells within the PBMC population. Overall, these findings underscore the potential of MR1 beads as effective tools for specific and targeted MAIT cell expansion, serving as a possible way to optimise CAR-MAIT cell manufacturing protocols. It aligns with our goal of enhancing the precision and efficiency of MAIT cell activation during CAR-T cell manufacturing, marking a crucial step towards the development of robust and adaptable cellular immunotherapies.

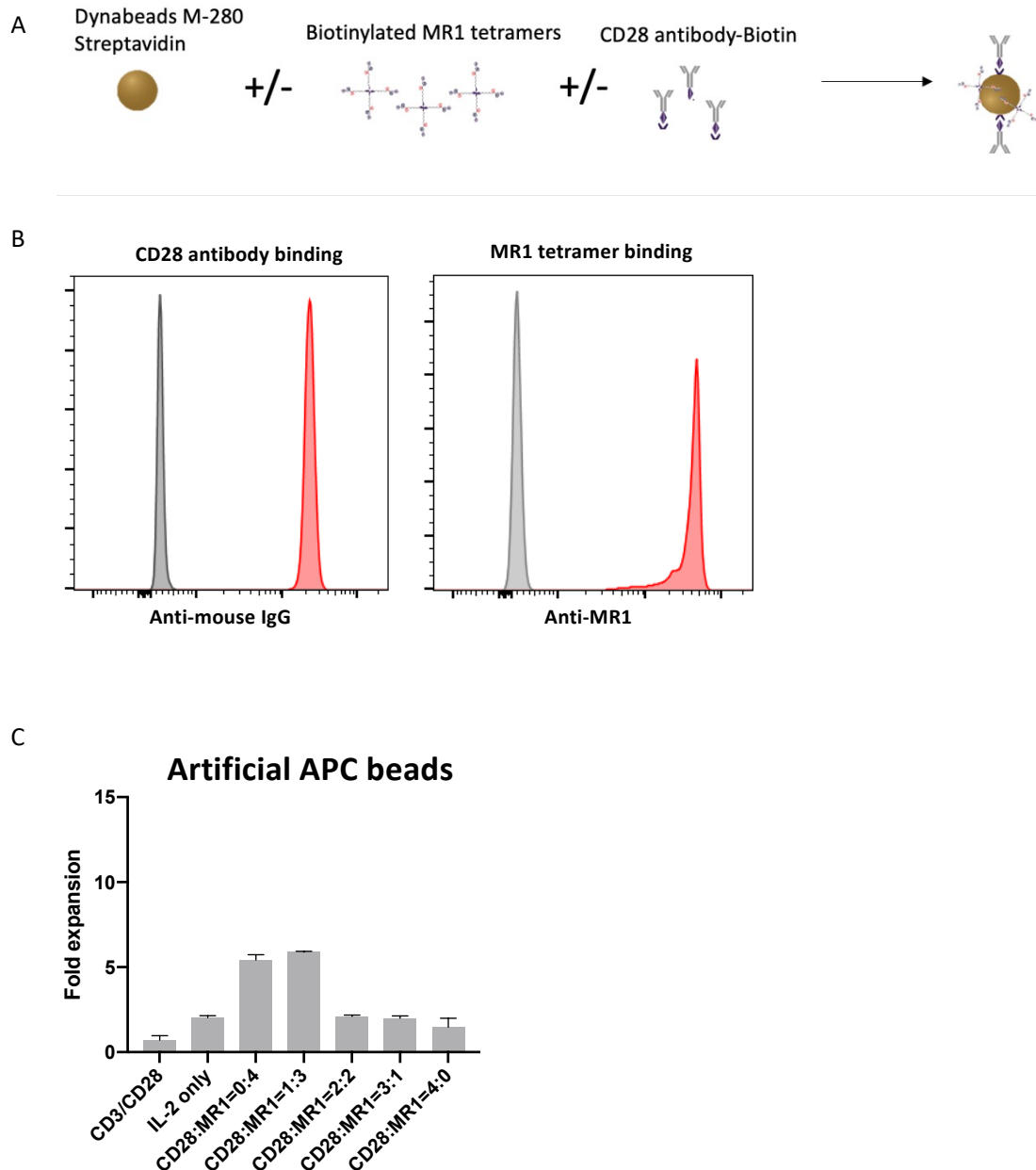


Figure 5.4. Generation of MR1 tetramer-based artificial APC beads for MAIT cell expansion.

(A) Schematic graph of MR1 tetramer-based artificial APC beads. The M-280 streptavidin Dynabeads were incubated with different ratios of 5-OP-RU-loaded human MR1 tetramers and an anti-human CD28 mAb. (B) Confirmation of the binding of anti-CD28 mAb and MR1 tetramers to the beads was achieved via flow cytometry using monoclonal antibodies against mouse IgG and MR1, respectively. In the flow cytometry plots, the MR1 beads were represented by the red histograms, while the original streptavidin beads (grey histograms) served as the negative control. (C) Fold expansion of MAIT cells within PBMCs over a 6-day culture period with the MR1 beads. PBMC cultures were subjected to beads loaded with varying ratios of MR1 tetramers and an anti-human CD28 monoclonal antibody. The frequency of MAIT cells was assessed using flow cytometry on both day 0 and day 6, illustrating the impact of MR1 bead stimulation on the proliferation of MAIT cells. Data with error bars are represented as mean $\pm$ SEM. PBMCs from three different normal donors were used.

5.4. Cytokine combinations impacting MAIT cell expansion

The optimal expansion of MAIT cells is a critical factor in harnessing their therapeutic potential. After validating the effectiveness of TCR-dependent stimulations using 5-OP-RU and MR1 beads, it is imperative to determine the most effective cytokine combinations to promote the specific stimulation and growth of MAIT cells within PBMCs. Conventional T cells are typically expanded using IL-2, IL-7, and IL-15, but MAIT cells, uniquely responsive to infection, necessitate IL-12 and IL-18 for an effective response (Ussher et al., 2014). In addition, some recent findings suggest enhanced MAIT cell-mediated control of infection through the addition of IL-23 plus 5-OP-RU prompted an exploration of optimal cytokine combinations (Wang et al., 2019; Teng et al., 2015). To ascertain the most effective cytokine combination, PBMCs were stimulated with MR1 tetramer beads or 5-OP-RU alongside various cytokines for 6 days. The expansion fold of MAIT cells, quantified as the fold change in frequency relative to the initial frequency for each donor, was assessed. Flow cytometry was employed to measure the frequency of MAIT cells before and after stimulation. The use of either MR1 beads or 5-OP-RU did not result in a significant difference in MAIT cell fold change. However, diverse cytokine combinations demonstrated varying degrees of influence on the expansion of MAIT cells. Among 15 combinations tested, the inclusion of IL-12 and IL-18, with or without IL-23, emerged as superior options, outperforming other combinations (Figure 5.5). While IL-2 is known to stimulate MAIT cell growth, its application for specific MAIT cell expansion within PBMCs is suboptimal, given its propensity to induce the proliferation of non-MAIT T cells.

Both of IL-12 and IL-23 belong to the IL-12 family of cytokines (Teng et al., 2015). Their shared p40 protein subunit intrigued our interest, prompting a comparative analysis of the effects of IL-12 or IL-23 alone or in conjunction with IL-18 on MAIT cell expansion. The extended culture period, coupled with 5-OP-RU supplementation every 7 days, allowed us to observe the overall growth trend of MAIT cells, with flow cytometry analysis on days 6, 13, and 20 respectively. IL-12/IL-23 and IL-18, working synergistically with TCR triggering, were the most effective in promoting MAIT cell expansion compared to other cytokine combinations. No significant difference in the fold expansion of MAIT cells was discerned between the IL-12 + IL-18 and IL-23 + IL-18 combinations. Importantly, the inclusion of IL-18 played a dominant role, as neither IL-12 nor IL-23 alone demonstrated substantial efficacy to fuel MAIT cell growth in the absence of IL-18 (Figure 5.6 A and B). This could be attributed to the heightened expression of IL-18R α on resting MAIT cells (Fergusson et al., 2014). Illustrating the potency of this cytokine combination, a representative donor, when subjected to antigenic stimulation with 5-OP-RU in the presence of IL-12 and IL-18, exhibited a specific activation and expansion of MAIT cells in PBMCs. The MAIT cell frequency increased from 2% on day 0 to over 25% on day 6 and an impressive 55% on day 13 (Figure 5.6 C). However, beyond day 13, MAIT cells plateaued, indicating a saturation point in their expansion dynamics (Figure 5.6 A). These findings underscore the critical role of cytokine combinations in shaping an optimal environment for the specific and robust expansion of MAIT cells, laying the foundation for optimised protocols in CAR-MAIT cell manufacturing.

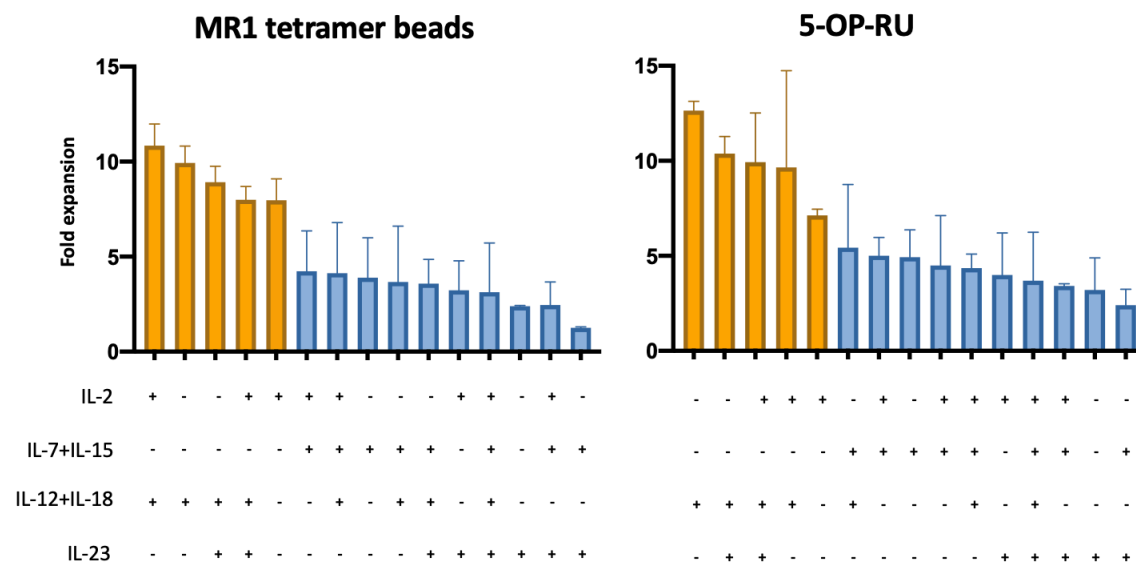


Figure 5.5. Effects of cytokines on MAIT cell expansion in short term.

Fold expansion of MAIT cells in PBMCs after 6-day stimulation with MR1 beads (cell to bead ratio = 1:1) or 100 nM 5-OP-RU in the presence of 15 different cytokine combinations. The frequency of MAIT cells was assessed using flow cytometry on both day 0 and day 6, illustrating the impact of cytokine combinations on the proliferation of MAIT cells. Data with error bars are represented as mean $\pm$ SEM. PBMCs from two different normal donors were used.

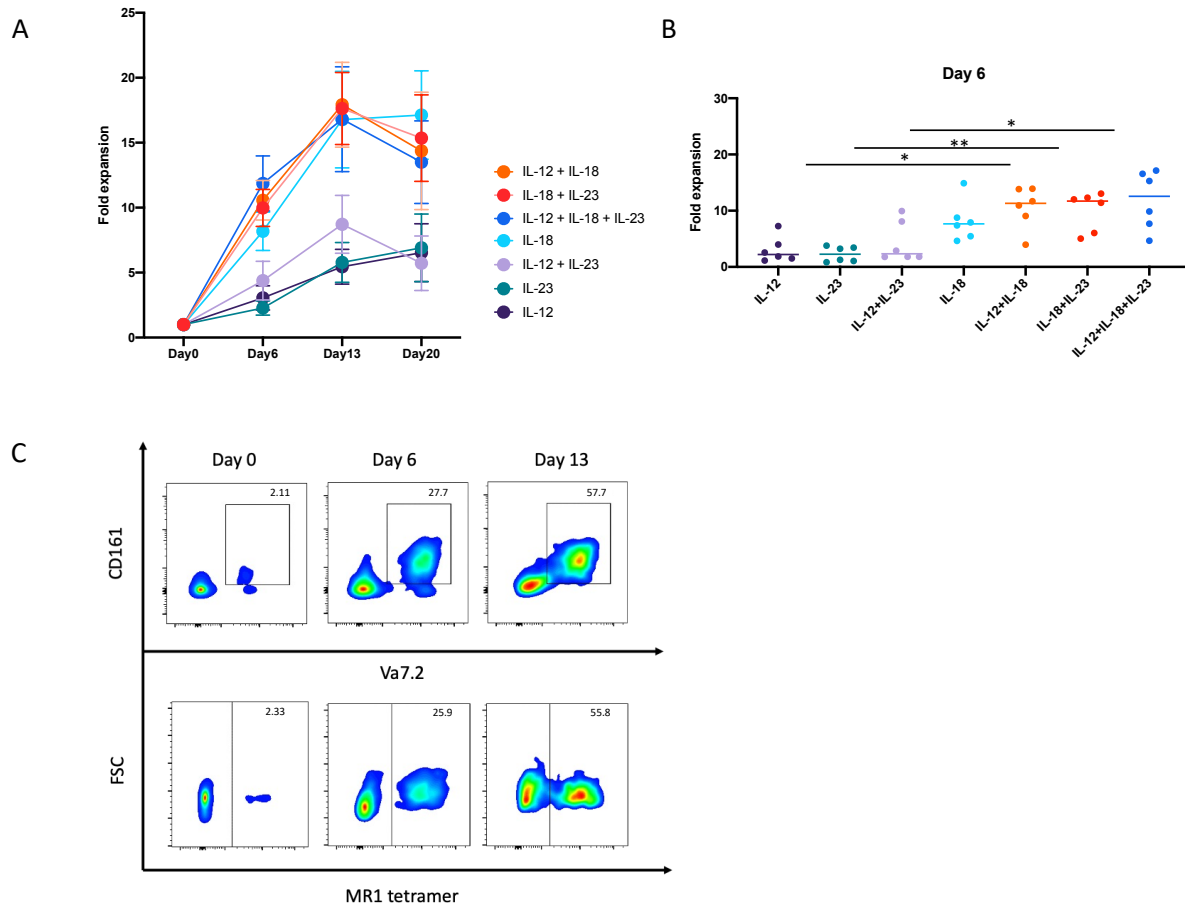


Figure 5.6. Effects of cytokines on MAIT cell expansion in long term.

(A) Fold expansion of MAIT cells in PBMCs on day 0, 6, 13 and 20 after stimulation with 5-OP-RU in the presence of 50 ng/mL IL-12, IL-18 and/ or IL-23. The frequency of MAIT cells was assessed using flow cytometry on day 0, day 6 and day 13, respectively, illustrating the impact of cytokine combinations on the proliferation of MAIT cells. PBMCs from 6 different normal donors (n=6) were used. (B) IL-18 combined with IL-12 or IL-23 has a synergized effect on MAIT cell expansion on day 6. The mean of MAIT cell fold increase on day 6 (n=6) was analysed across experiments by one-way ANOVA (*P < 0.05, **P < 0.01). Data with error bars are represented as mean $\pm$ SEM. (C) Representative graph of MAIT cell frequency change over 13 days under antigenic stimulation with 5-OP-RU in the presence of IL-12 and IL-18.

5.5. Effects of serum on MAIT cell expansion

When exploring ideal strategies for expanding MAIT cells, beyond TCR stimulation and cytokine selection, the effects of different serum conditions on MAIT cell growth were also investigated in this study. The aim was to identify serum formulations that would further support optimal and robust MAIT cell expansion while maintaining cell viability and minimizing exhaustion marker expression. PBMCs were cultured with 100 nM 5-OP-RU in the presence of IL-12 and IL-18, and the frequency change of MAIT cells was assessed after 6 and 13 days of culture. Evaluation was conducted using fetal bovine serum (FBS), human AB serum, and a novel xeno-free serum replacement product from Thermo Fisher, CTS™ Immune Cell SR. Interestingly, the use of the xeno-free serum replacement product yielded the highest expansion fold for MAIT cells. As exemplified by a representative donor, the initial MAIT cell frequency of 1.31% increased to 10.1% under FBS, 5.33% under human AB serum, and significantly rose to 21.6% under xeno-free serum replacement by day 6. By day 13, the frequency of MAIT cells cultured with xeno-free serum replacement could even increase to 55% (Figure 5.7). On average, MAIT cell expansion using xeno-free serum replacement exceeded 25-fold by day 13, showcasing the efficacy of the xeno-free serum replacement in promoting MAIT cell proliferation (Figure 5.8 A). This observation suggests that the components present in the xeno-free serum replacement may provide essential factors that enhance the expansion of MAIT cells more effectively than traditional serum formulations. Interestingly, no significant disparity in cell viability was observed across the three serum conditions, indicating that all serum formulations supported MAIT cell expansion without compromising cell survival (Figure 5.8 B). Upon harvesting the cultured cells, the expression levels of exhaustion markers PD-1 and TIM-3 on MAIT cells were assessed by flow analysis.

The use of the xeno-free serum replacement resulted in lower expression levels of these exhaustion markers compared to FBS and human AB serum (Figure 5.9 A and B). This suggests that the xeno-free serum replacement may provide a more conducive microenvironment for MAIT cell proliferation and maintenance, thereby mitigating the development of exhaustion phenotypes associated with prolonged *in vitro* culture.

The observed differential effects of serum compositions on MAIT cell expansion underscore the importance of serum selection in optimizing cell culture conditions for therapeutic applications. The significant enhancement in MAIT cell expansion achieved with the xeno-free serum replacement product suggests its potential as a superior alternative to traditional serum sources. The substantial increase in MAIT cell frequency, particularly evident by day 13, highlights the robust proliferative capacity of MAIT cells under favourable culture conditions. The consistent viability of MAIT cells across serum conditions implies that the observed differences in expansion are primarily attributed to variations in growth-promoting factors of the serum components. This validates the suitability of the xeno-free serum replacement product for supporting robust MAIT cell expansion without compromising cell viability. The reduced expression levels of exhaustion markers PD-1 and TIM-3 in MAIT cells cultured with xeno-free serum replacement further substantiate its favourable impact on maintaining MAIT cell functionality and fitness during expansion. These findings hold promise for advancing the development of MAIT cell-based therapies, as efficient expansion protocols are crucial for generating sufficient cell numbers for clinical applications.

In conclusion, optimizing culture conditions represents a pivotal aspect of MAIT cell manufacturing protocols. The noteworthy efficacy observed with stimulation using 5-OP-RU

in conjunction with IL-12 and IL-18 within the medium supplemented with xeno-free serum replacement represents a significant advancement in promoting MAIT cell proliferation. This approach not only fosters robust cell expansion but also preserves cell viability while mitigating the expression of exhaustion markers, highlighting its potential as a preferred choice for supporting MAIT cell-based therapies. By offering insights into refining manufacturing protocols for MAIT cell-based immunotherapies, these findings contribute significantly to advancing the field and accelerating the translation of MAIT cell-based treatments into clinical practice.

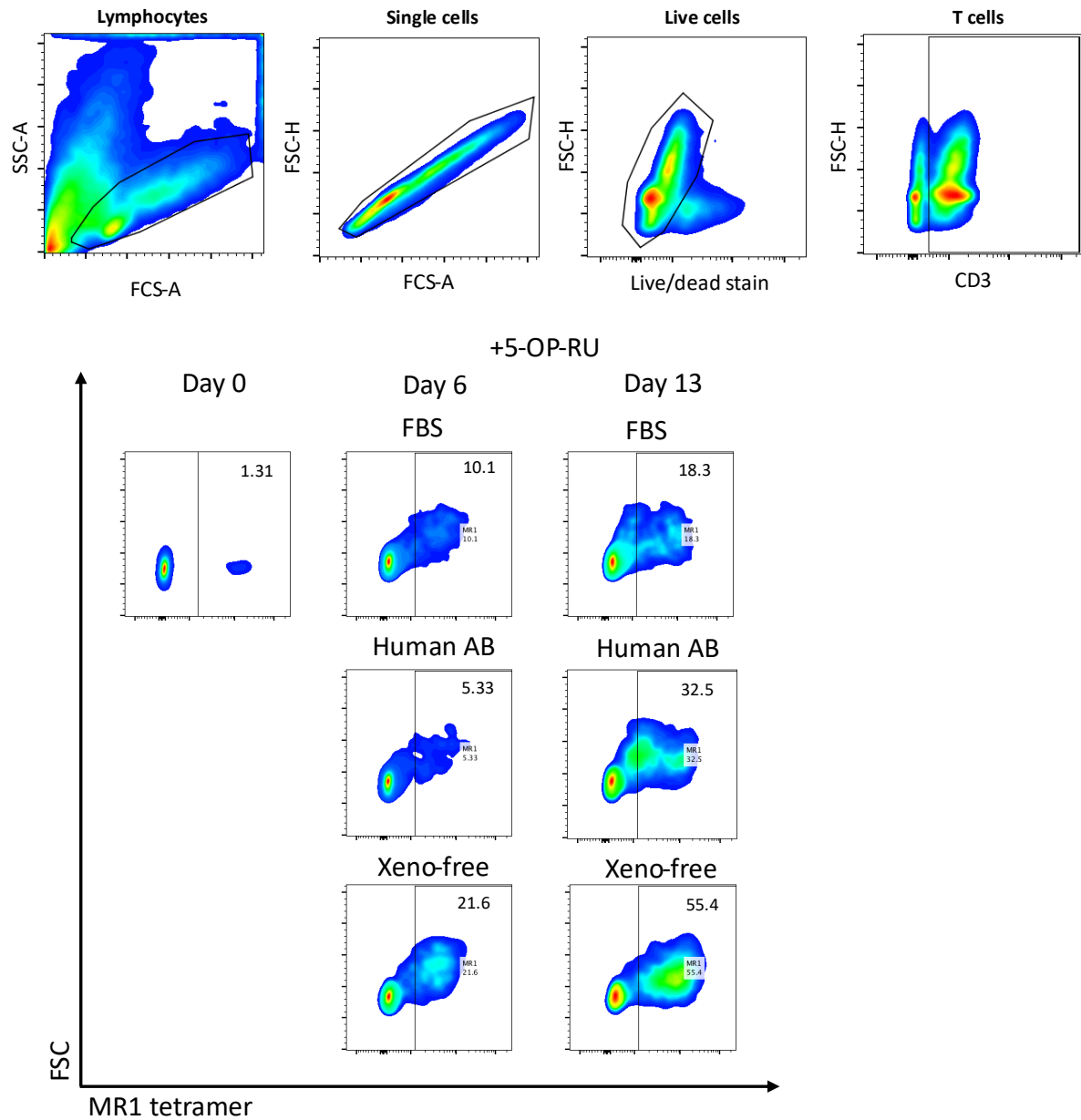


Figure 5.7. Effects of various serum compositions in the culture medium on MAIT cell growth over time.

Gating strategy and representative flow cytometry plots depicting the frequency change of MAIT cells after 6 and 13 days of stimulation with 100 nM 5-OP-RU in the presence of IL-12 and IL-18, under different serum conditions (FBS, human AB serum, and xeno-free serum replacement). MAIT cells were defined as CD3⁺ MR1 tetramer⁺ cells. Live and dead aqua staining was used to distinguish live and dead cells. The frequency of MAIT cells was assessed using flow cytometry on day 0, day 6 and day 13, respectively, illustrating the impact of serum on the proliferation of MAIT cells.

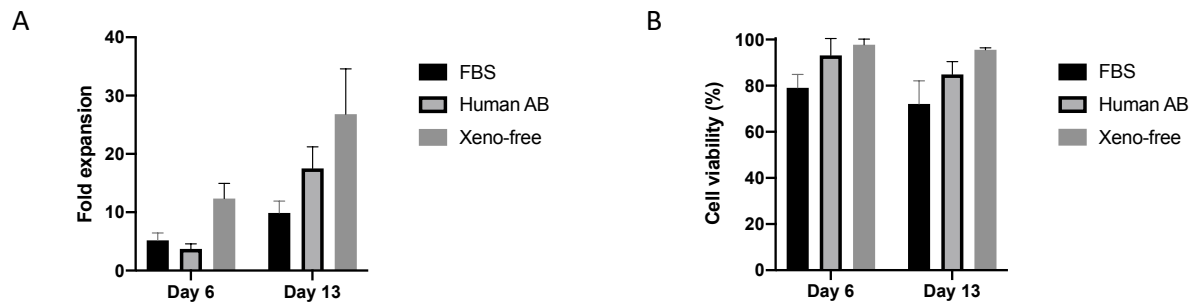


Figure 5.8. Serum effects on MAIT cell expansion fold and cell viability.

(A) Fold expansion of MAIT cells within PBMCs under various serum conditions, including FBS, human AB serum, and xeno-free serum replacement. (B) Assessment of cell viability under the influence of different serum conditions during the culture period. Data with error bars are presented as mean $\pm$ SEM. The study involved PBMCs from three distinct normal donors.



Figure 5.9. Serum effects on the expression of immune checkpoint on MAIT cells.

(A) Mean Fluorescence Intensity (MFI) analysis of PD-1 expression and (B) TIM-3 expression on MAIT cells. MFI values were measured in MAIT cells cultured under different serum conditions, analysed via flow cytometry on day 13. Data with error bars are presented as mean $\pm$ SEM. The study involved PBMCs from three distinct normal donors.

5.6. Discussion

The interest in developing an optimised culture protocol for MAIT cell expansion is driven by the imperative to address the challenges posed by the low frequency of MAIT cells within human PBMCs. Traditional approaches, such as isolating a small number of purified MAIT cells from blood and stimulating them with CD3 beads, are hampered by the requirement for large volumes of blood and labour-intensive procedures. In contrast, the MAIT cell-specific expansion strategy offers a more efficient and scalable solution. By leveraging this approach, smaller quantities of blood can serve as starting material for manufacturing, circumventing the need for extensive cell processing procedures and reducing resource consumption. The approaches include the use of specific stimulants, anti-V α 7.2 antibody, 5-OP-RU, artificial APC beads loaded with MR1 tetramers, and a careful selection of cytokine combinations.

The successful application of antibodies to stimulate T cell proliferation has revolutionized T cell culture, particularly in the expansion of unconventional T cell subsets such as $\gamma\delta$ T cells using anti-TCR $\gamma\delta$ antibodies. These expanded cells exhibit potent cytotoxicity against various tumour cells, underscoring the potential of antibody-based strategies in cancer immunotherapy (Kang et al., 2009; Zhou et al., 2012). However, the application of antibodies to selectively activate MAIT cells, characterized by their expression of the V α 7.2 TCR, remains a topic of ongoing investigation. The findings from the antibody stimulation experiments shed light on the complex interplay between MAIT cells and specific antibodies targeting the V α 7.2 antigen. Despite the initial hypothesis that anti-V α 7.2 antibodies would effectively stimulate MAIT cell expansion, the results demonstrated a surprising lack of significant proliferation within the MAIT cell population. Instead, the anti-V α 7.2 antibodies appeared to promote the

expansion of V α 7.2⁺ CD161⁻ non-MAIT cells. Several assumptions can be made to explain these unexpected findings. Firstly, it is plausible that V α 7.2⁺ CD161⁻ non-MAIT cells and MAIT cells utilize different signaling pathways in response to anti-V α 7.2 antibody stimulation. This could lead to preferential proliferation of one subset over the other. Differences in the expression or activation of signaling molecules involved in cell proliferation may exist between the two subsets. Variations in the intracellular signaling cascade downstream of TCR engagement could contribute to the differential expansion of V α 7.2⁺ CD161⁻ non-MAIT cells and MAIT cells. Besides, the microenvironment surrounding V α 7.2⁺ CD161⁻ non-MAIT cells and MAIT cells may influence their responsiveness to anti-V α 7.2 antibody stimulation. Factors such as cytokine profiles, co-stimulatory molecule expression, and interactions with other immune cells could impact their proliferation. Intriguingly, V α 7.2⁺ CD161⁻ non-MAIT cells exhibited significant proliferation upon stimulation with anti-V α 7.2 antibody, particularly when IL-15 was present, whereas MAIT cells demonstrated enhanced proliferation in the presence of IL-12 and IL-18. These findings underscore the potential divergent responses of these cell subsets to stimulatory signals. A recent RNA-seq analysis revealed that TCR α 7.2⁺ CD161⁻ T cells, while expressing the key marker TCR α 7.2 associated with MAIT cells, display distinct differences from MAIT cells in terms of gene expression. Despite the shared expression of TCR α 7.2, these cells exhibit gene expression patterns more closely resembling CD161⁻ conventional T cells rather than MAIT cells (Park et al., 2019). This finding underscores the significant divergence between TCR α 7.2⁺ CD161⁻ T cells and MAIT cells at the molecular level, highlighting their unique identity within the T cell landscape. This divergence in gene expression patterns between MAIT cells and TCR α 7.2⁺ CD161⁻ T cells may contribute to the differential response observed when stimulating these cell subsets with anti-V α 7.2 antibody. It is possible that the distinct gene expression profiles result in varied signaling pathways or

activation mechanisms in response to antibody stimulation, leading to differential proliferation or expansion outcomes. Further investigation into the specific molecular pathways and functional properties of TCR α 7.2⁺ CD161⁻ T cells compared to MAIT cells could provide additional insights into their differential responses to antibody stimulation. It is also a future research interest in exploiting the role of TCR α 7.2⁺ CD161⁻ T subsets in immune responses and how they are coordinated with MAIT cells. Given the limited impact of the antibody stimulation strategy on MAIT cell-specific expansion, this study proceeded to investigate the feasibility of alternative stimulation approaches.

In previous studies, PFA-fixed *E. coli* was employed to stimulate MAIT cells in PBMCs through antigen presentation by APCs (Hagel et al., 2020). Besides, direct injection of synthetic 5-OP-RU, in combination with other activation agents, has been shown to induce MAIT cell expansion in animal models to combat bacterial infection (Vorkas et al., 2020). Expanding upon these principles, the addition of the MR1 ligand 5-OP-RU directly into PBMCs could present an alternative approach, wherein host APCs present 5-OP-RU antigens to MAIT cells and induce MAIT cell-specific activation. Our results clearly confirmed the potency of 5-OP-RU in stimulating MAIT cells *in vitro*. Through its binding to MR1 on natural APCs in PBMCs, 5-OP-RU facilitated exclusive stimulation of MAIT cells, demonstrating an average ten-fold expansion within PBMCs upon 6 days of culturing. In addition to employing the 5-OP-RU antigen, the incorporation of MR1 beads as artificial APCs represents an alternative strategy for specific MAIT cell stimulation. These beads, composed of streptavidin coupled with biotinylated 5-OP-RU-loaded human MR1 tetramers and/or anti-human CD28 monoclonal antibody, offered controlled and tangible stimulation. Their use addresses the challenges associated with natural APCs, providing a substitute means for MAIT cell activation. This bead-

based approach also ensures a more straightforward removal process during manufacturing, streamlining the production of MAIT cell-based therapies. In addition, our exploration of cytokine combinations unveiled the critical role of IL-12, IL-23, and IL-18 in promoting optimal MAIT cell expansion. While IL-2, IL-7, and IL-15 are conventionally used for expanding T cells, this study underscores the unique cytokine requirements of MAIT cells. The synergistic action of IL-12 and IL-23, in conjunction with IL-18, emerged as the most effective combination for sustained MAIT cell growth. These findings align with existing literature, emphasizing the synergy of IL-12 with IL-18 in inducing IFN- γ production and amplifying TCR-mediated signals (Jesteadt et al., 2018; Leng et al., 2019). This not only sheds light on the nuanced regulatory mechanisms governing MAIT cell expansion but also provides a roadmap for refining manufacturing protocols for CAR-MAIT cell therapies. The optimised cell culture method ensures the selective activation and expansion of MAIT cells within the complex milieu of PBMCs, effectively addressing the challenge posed by their relatively low frequency in the circulating T cell pool. By employing this targeted stimulation approach, robust expansion of MAIT cells was achieved while mitigating the proliferation of other non-MAIT cells.

In addition, our examination of various serum supplements revealed intriguing insights into their impact on MAIT cell growth. While FBS and human AB serum are commonly used in cell culture, the introduction of a novel xeno-free serum replacement product, CTS™ Immune Cell SR, emerged as a promising alternative. Culturing MAIT cells with this xeno-free product led to significantly higher fold expansion compared to traditional serum options. Importantly, this enhanced expansion did not compromise cell viability, underscoring the suitability of the xeno-free serum replacement for supporting robust MAIT cell proliferation. Besides, the assessment of exhaustion markers PD-1 and TIM-3 revealed notable differences in their

expression levels under different serum conditions. Cultures supplemented with the xeno-free serum replacement exhibited lower expression levels of these exhaustion markers compared to FBS and human AB serum. This observation suggests that the choice of serum supplement may influence the immunophenotype of expanded MAIT cells, potentially impacting their functional properties and therapeutic efficacy. The effectiveness of this xeno-free serum replacement product, CTS™ Immune Cell SR, has been previously assessed *in vitro* for the expansion and lentiviral transduction of polyclonal T cells. Importantly, the study demonstrated that the xeno-free serum replacement supplemented medium facilitated optimal expansion of tumour antigen-specific T cells and promoted T cells to display a central memory phenotype. This phenotype is advantageous for *in vivo* survival and persistence following adoptive transfer. Besides, T cells expanded using the xeno-free medium exhibited high compatibility with lentivirus-mediated gene transduction, suggesting its potential application for culturing gene-modified T cells (Okern et al., 2015; Smith et al., 2015). These findings collectively underscore the utility of the xeno-free replacement product as a platform strategy for the manufacture of clinical-grade adoptive cellular therapies. Besides, the adoption of serum-free or xeno-free culture conditions eliminates the variability associated with animal-derived serum supplements and reduces the risk of immune reactions or contamination, further optimising the manufacturing process (Karnieli et al., 2017).

Overall, the MAIT cell-specific expansion strategy represents a promising approach for overcoming the inherent challenges of manufacturing MAIT cell-based therapeutics. These stimulation methods prove instrumental in eliciting a robust and specific response from MAIT cells, making it a groundwork in the development of optimised manufacturing protocols tailored to the unique requirements of MAIT cell-based therapies. The specificity imparted by

5-OP-RU and MR1 beads, combined with the synergistic cytokine milieu and the use of xeno-free serum replacement, not only maximize MAIT cell growth but also maintain favourable immunophenotypic characteristics. As we continue to unravel the intricacies of MAIT cell biology, these insights pave the way for the development of effective MAIT cell-based therapies with broad implications for the field of cellular immunotherapy.

Chapter 6. Final discussion

The field of CAR technology has witnessed significant advancements over the past few decades, accompanied by the development of various generations of CAR-based cell products designed for anticancer purposes. Currently, autologous-modified CAR-T cells represent the foundation of cell-based immunotherapy. Extensive clinical trials and research findings have underscored the potent anti-tumour effects and promising outcomes of CD19-specific CAR-T cells in autologous settings for the treatment of lymphocytic leukaemia, highlighting the enormous potential of CAR technology as a novel anticancer platform (Enblad et al., 2018; Frigault & Maus, 2020; Chow et al., 2019). However, despite the remarkable progress, the use of conventional T cells as the backbone for CAR therapy continues to face numerous challenges and limitations that hinder its widespread application. These challenges include the complexities of the tumour microenvironment, tumour antigen heterogeneity, limited organ infiltration ability, concerns regarding toxicity profiles, *in vivo* persistence of CAR-T cells and lower efficacies against solid tumours compared with haematological malignancies (Challener, 2016; Labanieh, Majzner & Mackall, 2018). Additionally, the intricate manufacturing procedures and high production costs associated with CAR-based therapy have restricted its accessibility. It is imperative to address these challenges and limitations to unlock the full therapeutic potential of CAR-based cell therapy. In this context, novel approaches and innovative strategies are being explored to enhance the efficacy, safety, and accessibility of CAR-T cell therapy, allowing its broader adoption and utilization in the fight against cancer.

The pursuit of developing novel "off-the-shelf" CAR-modified cell products with nonclassical T cell subsets represents a compelling strategy to overcome some of these hurdles. Allogeneic T cell products derived from healthy donors offer a viable solution to the challenges associated with autologous therapies, such as lengthy manufacturing processes and variability in patient response. By establishing a cell bank sourced from healthy donors with carefully selected biological parameters, the quality and consistency of the final product can be ensured, thereby facilitating broader access to therapy due to lower production costs enabled by scale-up manufacturing. However, the adoption of allogeneic therapy introduces significant concerns regarding the risk of GVHD and host rejection. Currently, the innate-like T cells, $\gamma\delta$ T cells and iNKT cells, are being explored as alternatives due to their unique antigen recognition mechanism independent of MHC, thus avoiding GVHD in allogeneic settings (Rotolo et al., 2018; Wang, 2018). Similarly, MAIT cells also do not rely on the MHC class I molecule for antigen recognition and are therefore less likely to induce allogeneic adverse reactions. Besides, MAIT cells have the advantage of being more abundant in the human immune system compared to $\gamma\delta$ T cells and iNKT cells, and they have natural tropism to mucosal tissues (Wang, 2018; Chiba, Murayama & Miyake, 2018). In this regard, MAIT cells hold significant potential to emerge as the one of the primary cell types in the future of adaptive off-the-shelf cancer immunotherapies.

MAIT cell research is a relatively new field in immunotherapy that our study is one of the very early efforts to modify MAIT cells with CAR and evaluate their efficacy. The key outcomes of this study were the efforts to optimise the manufacturing protocols for CAR-MAIT cells and demonstration that engineered CAR-MAIT cells perform cytotoxicity against specific cancer targets but show limited alloreactive response to the allogeneic stimuli. Collectively, the study

aimed at exploring the therapeutic potential of CAR-MAIT cells in the context of cancer immunotherapy.

The work started with the establishment of a third generation CD19-specific CAR construct. The scFv derived from the FMC63 monoclonal antibody served as a solid and reliable foundation for evaluating the functional efficacy of MAIT cells in targeting B cell-related malignancies due to its proven specificity and high binding affinity to CD19 (Zola et al., 1991; Kramer et al., 2016). The incorporation of multiple co-stimulatory domains, including CD28 and 4-1BB, was strategically implemented to enhance CAR-mediated signaling pathways, thereby promoting the activation, survival, and persistence of CAR-MAIT cells (Labanieh, Majzner & Mackall, 2018). This CAR construct also featured the integration of a truncated human EGFR as a detection marker. The flow cytometry analysis confirmed the successful co-expression of CAR and tEGFR marker. Efficient delivery of CAR genes into MAIT cells stands as a pivotal prerequisite for the successful generation of CAR-MAIT cells. To achieve this objective, an array of viral vectors, encompassing retroviral and lentiviral vectors, were engineered, and established for the transduction of MAIT cells with CAR constructs. The retrovirus platform employed a two-step transduction protocol involving Plat-GP and PG13 cells as producer cell lines to package the CAR-expressing virus. On the other hand, the lentivirus platform utilized the 293T cell line to enable efficient virus production with elevated virus titer. By establishing both systems, the thesis ensured effective transduction capabilities across diverse settings and conditions. The retrovirus platform takes the advantage of inducing stable and enduring CAR expression, a feature crucial for ensuring sustained therapeutic efficacy. The development of a stable virus packaging cell line enables constitutive viral particles production. Meanwhile, the lentivirus platform, with its capacity

for higher virus titer, facilitates scenarios requiring precise modulation and high levels of transduced cells. These two platforms significantly enhance the adaptability and versatility of CAR-T cell therapy, establishing a comprehensive toolkit to address a spectrum of research contexts and therapeutic imperatives. In future endeavours, establishing lentivirus stable cell lines could facilitate scalable lentivirus production for manufacturing purposes. In this phase of the study, MAIT cells were extracted from human PBMCs using MACS isolation techniques and activated by the broad-spectrum CD3/CD28 Dynabeads in conjunction with IL-12 and IL-18 prior to transduction. This study optimised the virus-based CAR transduction protocols to maximize transduction efficiency while concurrently preserving MAIT cell viability and functionality. Overall, the optimization process underscores the commitment to ensuring robust and reliable CAR-MAIT cell generation, thereby ensuring the translational potential and clinical applicability of CAR-MAIT cell therapy.

In following part of the study, the transduced CAR-MAIT cells underwent evaluations to determine their efficacy and therapeutic potential in targeting and eliminating specific target cells *in vitro*. Characterization of the phenotypical features of MAIT cells revealed distinctive surface marker expression profiles and functional properties. MAIT cells predominantly exhibited a CD8⁺ phenotype, underscoring their cytotoxic potential. Further investigation into memory effector markers, CD45RO⁺, CD45RA⁻, CD62L⁻ and CCR7⁻, and tissue homing markers, CCR5 and CCR6, revealed insights into their enhanced capacity for rapid response and efficient migration to peripheral tissues, facilitating targeted effector functions. This suggests an improved ability to infiltrate and target tumour cells within solid tissues, potentially enhancing therapy efficacy. Besides, the bias of MAIT cells toward an effector memory state implies not only immediate cytotoxic effects but also long-term persistence in the host,

fostering prolonged tumour surveillance and control (Golubovskaya & Wu, 2016). Functional characterization of CAR-MAIT cells highlighted their robust cytotoxic activity against CD19-expressing target B cell lines. In the 20-hour *in vitro* killing assay, CAR-MAIT cells lysed approximately 70-80% of the target BLCLs at a 5:1 effector-to-target cell ratio, demonstrating their potential as a novel candidate for CAR-based immunotherapy. The cytotoxicity displayed by CAR-MAIT cells was comparable to conventional CAR-T cells, emphasizing the effectiveness of CAR-MAIT cells and their potential as the alternative therapeutic approach. The potential side effects and efficacy of CAR-MAIT to eradicate tumour targets in the *in vivo* models needs to be assessed in the future. Despite strong cytotoxic capacity, a recent study has demonstrated that CAR-MAIT cells secrete lower levels of cytokines, including IFN- γ , Granzyme B, and GM-CSF, compared to conventional CAR-T cells (Dogan et al., 2022). This reduced cytokine release may enhance safety by mitigating the risk of T cell overactivation and associated adverse effects like cytokine release syndrome. In particular, lower GM-CSF levels may reduce neuroinflammation while improving CAR-T cell function (Stern et al., 2019). Additionally, investigation into the alloreactive potential of MAIT cells elucidated their unique properties and suitability for allogeneic cellular therapy. The *in vitro* MLR assay demonstrated that MAIT cells exhibited low alloreactivity when exposed to allogeneic stimuli, prompting their use as universal cells for allogeneic immunotherapy. Taken together, these findings support the idea that CAR-MAIT cells could be a novel avenue to produce the off-the-shelf cellular therapeutics with reduced risk of immune-mediated adverse reactions.

The challenges of developing CAR-MAIT cell therapy include the difficulty of expanding cells to a sufficient level for clinical application and standardization of the production protocol. Given that MAIT cells constitute a relatively small proportion of the total T cell population, a

key focus was establishing optimal MAIT cell-specific expansion assays to facilitate large-scale CAR-MAIT cell production. In this study, multiple MAIT cell-specific expansion strategies were investigated and refined beyond the conventional CD3-based activation approach. First, the unexpected outcomes arising from the utilization of anti-V α 7.2 antibodies to prompt MAIT cell proliferation highlight the complexities of MAIT cell activation and expansion. While initial hypotheses proposed effective and selective stimulation of MAIT cell proliferation via the anti-V α 7.2 antibodies, the results unveiled a surprising lack of significant expansion within the MAIT cell population. Instead, the anti-V α 7.2 antibodies appeared to foster the proliferation of V α 7.2⁺ CD161⁻ non-MAIT cells. Further exploration of the specific molecular pathways and functional properties of the two cell subsets is warranted, as it could provide valuable insights into their distinct responses to antibody stimulation. Expanding on this, alternative strategies were employed to further explore the method for MAIT cell-specific expansion. Results demonstrated that 5-OP-RU stimulation induced significant expansion of MAIT cells within PBMCs in a dose-dependent manner, with concentrations at 100 nM and 1000 nM yielding over a ten-fold increase in MAIT cell population after 6 days of culture. Additionally, artificial MR1 beads were developed as alternative antigen-presenting platforms for MAIT cell stimulation, showcasing their potential for specific MAIT cell expansion. These beads comprise streptavidin beads coupled with biotinylated 5-OP-RU-loaded human MR1 tetramers and anti-human CD28 monoclonal antibody at varying ratios. They exhibited enhanced efficacy to promote MAIT cell growth, particularly at CD28:MR1 ratios of 0:4 and 1:3. The impact of cytokine combinations on MAIT cell expansion was explored to determine optimal culture conditions for CAR-MAIT cell manufacturing. The findings highlighted that IL-18, supplemented with IL-12 or IL-23, significantly augmented MAIT cell expansion compared to other cytokine combinations, thereby offering an optimised protocol for robust and

selective MAIT cell expansion for enhanced CAR-MAIT cell therapy. Alongside, the influence of different serum conditions on MAIT cell growth was investigated to further improve the protocol for CAR-MAIT cell expansion. The xeno-free serum replacement product markedly boosted MAIT cell expansion compared to FBS and human AB serum, underscoring the pivotal role of serum selection in refining culture conditions for CAR-MAIT cell manufacturing. The harvested CAR-MAIT cells showed a less exhausted phenotype, indicating enhanced functionality and prolonged survival. These features are highly relevant for the potential clinical application of CAR-MAIT therapies. Besides, using xeno-free culture conditions offers additional advantages for standardized CAR product manufacturing by eliminating variability associated with serum supplements derived from different batches and reducing the risk of contamination. In summary, through optimization of TCR-dependent stimulation and cytokine combinations, robust expansion of MAIT cells while mitigating the proliferation of non-MAIT cells was achieved, thus laying a solid foundation for the scalable manufacturing of CAR-MAIT cell products.

While this study has provided encouraging results and valuable insights into the potential of CAR-MAIT cells as a novel avenue for cancer immunotherapy, numerous works for future research and development remain to be explored. Firstly, further investigations are necessary to fully understand the long-term safety profile and potential adverse effects linked with CAR-MAIT cell therapy, especially concerning off-target effects and immune-related toxicities. Evaluating their efficacy in allogeneic settings within humanized animal models and clinical trials to eradicate tumours while mitigating the risk of inducing GVHD will be crucial. On the other hand, it is also important to develop strategies to enhance allogeneic cell *in vivo* persistence and reduce allorejection for advancing the universal CAR-MAIT cell approach. One

approach to avoid rejection of allogeneic CAR T cells involves establishing a bank of cells that match the majority of the population in terms of HLA alleles. Experience from organ transplantation has shown that with the availability of suitable donors, a cell bank comprising T cells from 150 selected homozygous HLA-typed volunteers could match 93% of the UK population (Taylor et al., 2012). Besides, during the manufacturing of CAR-MAIT cells, gene editing of MHC class I molecules, such as disrupting the β 2-microglobulin locus on T cells, might help mitigate the risk of rejection by the host immune system (Depil et al., 2020). Further optimising the manufacturing processes to enhance CAR-MAIT cell expansion and functionality will be essential for realizing their full therapeutic potential. At the present stage, the overall CAR-MAIT production level is limited to the research scale. In the further works, we will explore the feasibility of applying the G-rex cell culture system and automated *CliniMACS Prodigy* system to assess if a clinical scale of cell products could be made under the GMP compliance. These two systems have been fully examined in manufacturing the conventional CAR-T cell products for clinical use (Zhu et al., 2016; Shah et al., 2017). Besides, exploring biomarkers for the selection of optimal healthy blood donors for the engineering and expansion of MAIT cells is imperative and could be one of focuses of our future study. Throughout this thesis, initial MAIT cell frequencies from various donors ranged from 1% to 8%. Although donors with higher initial frequencies may yield purer MAIT cell populations more rapidly, no significant difference was observed in the final harvest numbers. This finding aligns with current research indicating that despite significantly lower MAIT cell frequencies in the peripheral blood of non-small cell lung cancer (NSCLC) patients compared to healthy donors (median 0.9% vs. 5.79%), no notable difference in MAIT cell proliferation, apoptosis, expression of chemokines/receptors was observed between these individuals (Shi et al., 2023). Investigating other biomarkers correlating with MAIT cell functionality could offer

predictive insights into their therapeutic efficacy. Besides, our future studies will continue evaluating the efficacy of CAR-MAIT cells in diverse tumour models. Here, this study only elucidates the efficacy of CAR-MAIT cells equipped with the CD19-specific 3rd generation CAR construct. In the future works, MAIT cells could be modified with new generations of CARs, such as the novel split CAR system that contains the universal receptor and corresponding specific adaptor as introduced in the introduction part of the thesis for adjustable scFv pairing. Since MAIT cells have been demonstrated to obtain tissue-homing capacity, the *in vivo* study could also monitor if CAR-MAIT cells could effectively infiltrate the tumour environment, especially the mucosal-associated solid tumours. These outcomes will be essential for informing subsequent clinical trials. Overall, continued research efforts and collaborations are needed to overcome current challenges and realize the promise of CAR-MAIT cell therapy as a transformative approach in cancer treatment.

In conclusion, this study represents a significant step forward in cancer immunotherapy, showcasing the feasibility and effectiveness of CAR-MAIT cells as an innovative therapeutic approach. By combining advanced CAR technology with the distinct properties of MAIT cells, CAR-MAIT cells offer promising solutions to existing therapeutic challenges and open avenues for the development of more potent, accessible, and widely applicable CAR-based cell therapies. These findings add to the expanding body of evidence supporting the utility of unconventional T cell subsets in adoptive cell therapy and set the stage for continued research and clinical translation in this dynamic field. Further exploration through future benchwork and clinical trials will be imperative in fully unlocking the therapeutic potential of CAR-MAIT cells and integrating them into the forefront of immunotherapy.

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