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Approaches to enhance CAR T cells for use in inhospitable disease environments

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Approaches to enhance CAR T cells for use in inhospitable disease environments

Thesis for Doctoral Degree (Ph.D.)

By

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For Santo and Ghita Micallef

‘O, ljuvlighet, att när man har ätit upp allt, druckit ur allt, talat om allt och dansat sina ben trötta gå hem i den tysta timmen före soluppgången för att sova.’

Tove Jansson, från *Trollkarlens hatt* (1948)

Popular science summary

Cancer is an endlessly complex group of diseases that accounted for 9.7 million deaths in 2022,[1] representing approximately one out of every seven deaths globally. The gold standard for treatment varies between diseases but usually involves some combination of surgery, chemotherapy, and radiotherapy. Despite significant advances over the last decades, most forms of cancer are incurable, either unresponsive to treatment or, after an initial period of remission which may last for years, subject to disease recurrence. Immunotherapy, or using the immune system to fight cancer, is considered to have originated in 1890s, when a surgeon named William Coley, now known as the Father of Cancer Immunotherapy, discovered that treating cancer patients with certain bacterial strains appeared to enhance antitumor immunity.[2,3] In the 130-odd years since, members of the scientific community from all over the world have contributed to the incredible headway that has been made in the treatment of cancer utilizing immunotherapy.

Broadly speaking, immunotherapy works in one of two ways: either by giving a patient genetically engineered immune cells, or by treating a patient with a drug that stimulates their own immune cells to function better. This thesis focuses on chimeric antigen receptor (CAR) T cells, which are a patient's own T cells that have been genetically modified to express a receptor that allows them to target specific markers visible on the surface cancer cells. CAR T cells are commercially available for a variety of blood cancers, including leukemias and lymphomas, and have completely revolutionized the treatment of blood cancers that either do not respond to treatment, or in diseases that initially respond but then relapse. Before CAR T cells, patients who did not respond or whose disease came back after initially being eradicated, had no curative treatment options. Today, CAR T cells can cure approximately half of them.[4] There are many reasons why CAR T cells only work in half of the cases, including if the disease itself can no longer be targeted by CAR T cells, or if the quality of the CAR T cell product is poor. Another significant challenge has been solid tumors – blood cancers, even ones that create "zones" of tumor tissue within, for instance, lymph nodes, are regarded as liquid tumors. Solid tumors are often surrounded by a scaffold connective tissue and cells that form a protective shell around the tumor; inside the tumor, there is extremely limited access to the oxygen and nutrients CAR T cells need to survive and function. Further, the interior of a tumor can contain cells that dampen CAR T cell activity, providing yet another hurdle in an already incredibly hostile environment.

In **Paper I**, we focused on understanding how cancer cells are able to "hide" from CAR T cells through trogocytosis, where CAR T cells actually nibble the cell surface markers off tumor cells, making the tumor cells invisible to CAR T cells. The CAR T cells will then "wear" the cell surface marker on their own surface, leading to them being targeted and killed by other CAR T cells. We used two different CAR T cell structures (M28ζ and MBBζ) and found that both structures led to similar levels of trogocytosis, and that CAR T cells that did "steal" receptors from tumor cells often appeared less functional than CAR T cells that did not. CAR T cells are frequently combined with chemotherapy, as this has been shown to enhance patient outcomes. In **Paper II**, we tested a combination of drugs that has not previously been used in combination with CAR T cells, in order to see if the drugs would contribute to killing tumor cells, but at the same time not kill the CAR T cells themselves. We found that the combination of drugs was able to kill tumor cells and did not appear to have an outsize impact on the CAR T cells. Finally, in **Paper III,** we wanted to assess whether CAR T cells produced in an environment of restricted oxygen, like one might find in a tumor, performed better in a tumor-like environment. We found that producing CAR T cells in 2% O_2 rather than atmospheric oxygen levels (21% O_2) led to lasting changes in the CAR T cell makeup that might be favorable in the context of cancer treatment.

Ultimately, the goal of this thesis was to try to understand mechanisms that lead to CAR T cell therapy failure, and to examine different ways of improving them. CAR T cells have already been cemented as an important weapon in the fight against cancer, but many challenges still remain, several of them shrouded in darkness. Hopefully this thesis will help to shed a little bit of light on the questions we have yet to answer.

Abstract

Chimeric antigen receptor (CAR) T cell therapy has transformed the landscape of cancer treatment, curing diseases that were once incurable, including relapsed and refractory hematological malignancies. About half of the people treated with CAR T cells for B cell tumors either do not respond to treatment or experience disease relapse. Over the past several years numerous attempts have been made to understand why CAR T cell therapy fails when it does, and inroads have been made – albeit with limited success – into establishing CAR T cell therapy for solid tumors. In the three papers that make up this thesis, we sought to understand mechanisms that contribute to CAR T cell failure and explored approaches to enhance CAR T cells for use in inhospitable disease environments. In **Paper I,** we examined the mechanism of trogocytosis in an *in vitro* model of ovarian cancer using two different anti-mesothelin (MSLN) CAR T cell constructs, one with a CD28 (M28ζ) co-stimulatory domain, and the other with a 4-1BB (MBBζ) co-stimulatory domain. In **Paper II**, we evaluated the feasibility of combining the chemotherapeutic drugs Treosulfan (Treo) and Fludarabine (Flu) with three different anti-MSLN CAR T cell constructs in an *in vitro* model of ovarian cancer. Finally, in **Paper III**, we interrogated the impact of producing CAR T cells in low-physiological oxygen levels (2% O_2), to investigate whether production with restricted access to oxygen would lead to the preferential development of a CAR T cell compartment better equipped to function in the oxygen-restricted tumor microenvironment (TME) found in certain B cell malignancies, such as chronic lymphocytic leukemia (CLL).

List of scientific papers

I. Schoutrop E., Renken S., **Micallef Nilsson I.**, et al. (2022). Trogocytosis and fratricide killing impede MSLN-directed CAR T cell functionality. *Oncoimmunology,* 11(1), 2093426. doi: 10.1080/2162402X.2022.2093426.

II. El-Serafi, I., **Micallef Nilsson, I.**, Moter, A., et al. (2024). Impact of fludarabine and treosulfan on ovarian tumor cells and mesothelin chimeric antigen receptor T cells. *Cancer Immunol Immunother, 73*(9), 163. doi: 10.1007/s00262-024-03740-3.

III. **Micallef Nilsson, I.**, Poiret, T., Ryu, J., et al. (2025). Production of functional CD19 CAR T cells under hypoxic manufacturing conditions. *Front Immunol, 16,* 1675786. doi: 10.3389/fimmu.2025.1675786.

Contents

List of abbreviations

Akt	Protein kinase B
ACT	Adoptive cell therapy
APC	Antigen-presenting cell
CAF	Cancer-associated fibroblast
CAR	Chimeric antigen receptor
CIM	Co-inhibitory molecule
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte associated protein 4
Cr^{51}	$Chromium^{51}$
Cy	Cyclophosphamide
EOC	Epithelial ovarian cancer
ETC	Electron transport chain
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
Flu	Fludarabine
GZMB	Granzyme B
HIF	Hypoxia-inducible factor
ICI	Immune checkpoint inhibitor
ICS	Intracellular cytokine staining
IFNγ	Interferon γ
ITAM	Immunoreceptor tyrosine-based activation motif
LAG3	Lymphocyte activation gene 3
LDH	Lactate dehydrogenase
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MSLN	Mesothelin
MTG	MitoTracker Green
mTOR(C)	Mammalian target of rapamycin (complex)
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NK	Natural killer
NKT	Natural killer T

OXPHOS	Oxidative phosphorylation
PD-1	Programmed death-1
PD-L1	Programmed death-ligand 1
PI3K	Phosphatidylinositol 3-kinase
r/r	Relapsed/refractory
ROS	Reactive oxygen species
scFv	Short-chain variable fragment
TAM	Tumor-associated macrophage
TCR	T cell receptor
T_{CM}	Central memory T cell
T_{EM}	Effector memory T cell
T_{EMRA}	Effector memory T cell re-expressing CD45RA
TGFβ	Transforming growth factor β
TIL	Tumor infiltrating lymphocyte
TIM3	T cell immunoglobulin and mucin domain-containing protein 3
TME	Tumor microenvironment
TMRE	Tetramethylrhodamine ethyl ester
T_N	Naïve T cell
TNF	Tumor necrosis factor
TNFR	Tumor necrosis facor receptor
TRAF	TNFR-associated factor
T_{REG}	Regulatory T cell
T_{RM}	Tissue resident memory T cell
T_{SCM}	Stem cell memory T cell
Treo	Treosulfan
PARP	Poly-ADP-ribose polymerase
VEGF	Vascular endothelial growth factor

Introduction

Chimeric antigen receptor (CAR) T cells have transformed the landscape of treating relapsed and refractory (r/r) hematological malignancies. These specifically targeted, highly potent immunotherapeutic agents have transformed the landscape of treating hematological malignancies; however, results are inconsistent. To date, only about 50% of patients with hematological malignancies treated with CAR therapy go into durable remission, and none of the success in treating hematological malignancies has translated to solid tumor disease. Bridging this gap will require a more comprehensive understanding of the diseases we seek to treat, and improved insight into how CAR T cells behave in these disease environments. We have studied CAR T cells targeted against CD19, which is expressed on B cell malignancies, and mesothelin (MSLN), which is expressed on a variety of solid tumor types, including ovarian cancer. We have studied the impact of trogocytosis and fratricide killing in a model of $MSLN^{+}$ ovarian cancer, investigated the impact of two common chemotherapeutic agents on CAR T cells in order to assess the potential for combination therapy, and assessed CAR T cell responses to unfavorable growth environments, with limited access to oxygen.

1 Background

1.1 The immune system

The immune system is composed of an adaptive and innate branch which work together to fight disease. The innate branch mobilizes within hours to clear threats and is composed of a variety of cells that serve this purpose, including macrophages, dendritic cells, and granulocytes. The adaptive immune system, in contrast, requires activation from external signals to become activated and thus has longer response times compared to the innate. The adaptive branch is able to establish immune memory, allowing for a faster rate of response if re-exposed to the same pathogen. Cells of the adaptive immune system include T and B lymphocytes.[5,6] Some cells, including natural killer (NK) cells along with unconventional T cells such as natural killer T (NKT) and γδ T cells, have features of both innate and adaptive function. The cells of the immune system all derive from hematopoietic stem cells in the bone marrow and are of either lymphoid or myeloid lineage,[5] outlined in Figure 1.[7]

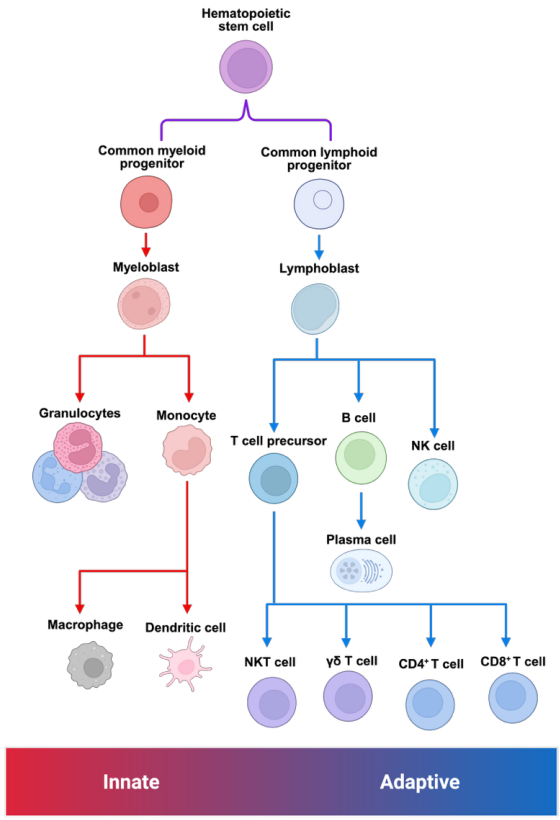

Figure 1. Cells of the immune system. Adapted from Murin et al Front immunol, 2020.

1.2 T cells

T cells are key effectors of the adaptive immune system. Conventional (αβ) T cells are broadly divided into either CD4⁺ "helper" or CD8⁺ "killer". Naming follows function, and so CD4⁺ cells "help" other cells of the immune system with their

respective functions, by for instance inducing antibody production by B cells, and CD8⁺ T cells kill diseased cells.[5]

1.2.1 The T cell receptor

T cells function through the engagement of the T cell receptor (TCR), a multiprotein complex composed of two TCR chains – α and β or γ and δ. The TCR is associated with the CD3 receptor complex, which is composed of six chains: the CD3εδ and CD3γε heterodimers, and the CD3ζζ homodimer. Each TCR chain is composed of a variable (V) and a constant (C) region, where the distal V region contains the antigen binding site, and the proximal C region comprises an immunoglobulin-like domain, connecting peptide, transmembrane region, and cytoplasmic tail.[8] The CD3γ, -ε, and -δ chains each contain a single immunoreceptor tyrosine-based activation motif (ITAM); each ζ chain contains three. ITAMs are important for T cell development and intracellular signal amplification.[9,10] The TCR-CD3 complex is shown in Figure 2.

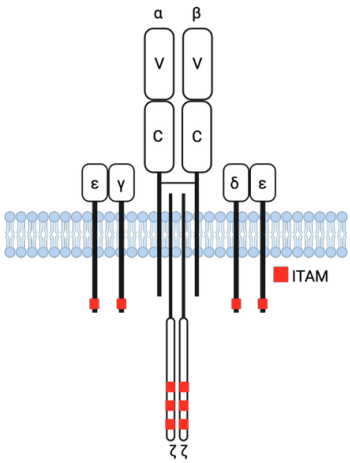

Figure 2. T cell receptor structure.

1.2.2 T cell activation

T cells require three signals to become fully activated. The first signal occurs when T cells recognize peptide antigens presented on antigen-presenting cells (APCs). When pathogens enter the bloodstream or tissues, they can be picked up and digested by local APCs. The APCs then digest the microbes into peptide sequences and travel to the secondary lymphoid tissues to present them to T cells. The peptides are displayed on major histocompatibility complex (MHC) molecules – either MHC class I, which is expressed by all nucleated cells, or MHC class II, which is expressed primarily by "professional" APCs – dendritic cells, macrophages, and B cells. CD4⁺ T cells recognize peptides displayed on MHC class II, and CD8⁺ T cells recognize peptides displayed on MHC class I. The second signal occurs when the T cell's CD28 co-stimulatory domain comes into contact with CD80 or CD86 receptors on an APC. The third signal is mediated by cytokines, which amplify the T cell response and orchestrate differentiation into specific subsets. T cell activation leads to clonal expansion and a massive T cell response tailored to a given antigen.[5]

1.2.3 CD4⁺ subsets

There are several CD4⁺ T-helper (T_H) subsets, each with different functions. Four which were of particular interest to us in the work presented here are T_H1, T_H2, T_H17, and regulatory T (T_{REG}). T_H1 cells are important effectors in the defense against intracellular pathogens, including bacteria and viruses, T_H2 react to allergens and defend against extracellular pathogens such as parasites, T_H17 function against extracellular pathogens, including certain bacteria and fungi, and T_{REG} cells participate in immune regulation. Each subset has also been shown to play a role in cancer. Each of these subsets is associated with a transcription factor and the production of specific cytokines; these are presented in Table 1.[11–13]

Subset	Transcription factor	Cytokines produced
T_H1	TBET	IFNγ, TNF
T_H2	GATA3	IL-4, IL-5, IL-13
T_H17	RORγT	IL-17, IL-23
T_{REG}	FOXP3	IL-10, TGFβ

Table 1. T helper subsets. IFNγ = interferonγ; TGFβ = transforming growth factorβ; TNF = tumor necrosis factor.

T_H1, T_H2, and T_H17 can be defined according to their surface expression of the chemokine receptors CCR4, CCR6, and CXCR3. T_H1 is $CCR4^-CCR6^-CXCR3^+$, T_H2 is $CCR4^+CCR6^-CXCR3^-$, and T_H17 is $CCR4^+CCR6^+CXCR3^-$. An additional subset that was of interest in this thesis is T_H1^*, which can be defined as $CCR4^-CCR6^+CXCR3^+$. These cells, first defined by Becattini and colleagues in 2015, produce high levels of IFNγ and low levels of IL-17.[14]

T_H cells have pleiotropic roles in cancer response. T_H1 are associated with positive outcomes in a variety of tumor types including ovarian cancer and glioblastoma, partly due to the effects of IFNγ, which include the inhibition of angiogenesis. Conversely, in some contexts IFNγ has been implicated as a mediator of immune escape,[11,15] suggesting a potentially pro-cancer role for T_H1 cells. T_H2 cells are widely regarded as having primarily pro-tumor effects, including through the induction of tumor angiogenesis; they have been correlated with poor

disease outcomes in several diseases, including ovarian cancer.. T_H17 cells are less well-defined, and their pro- and anti-tumor functions seem highly context-dependent.[11] The role of T_H1* in cancer has yet to be confirmed but is likely also pleiotropic, considering this subset's secretion of IFNγ. T_{REG} cells, with their inherent immunosuppressive function, are highly unfavorable in the context of the TME. They are specifically implicated in cancer-associated immune dysfunction through their ability to suppress effector T cell responses.[12]

1.2.4 CD8⁺ T cells

The hallmark of CD8⁺ T cells is their cytotoxicity. Binding of the TCR to the peptide-MHC complex of an infected cell forms an immunological synapse[16] through which the CD8⁺ T cell mediates killing, which occurs in two main ways. One is through secretion of cytotoxic molecules contained in intracellular granules[17] such as the cytokine granzyme B (GZMB), which activates enzymes that induce apoptosis, and the protein perforin,[18] which breaks down membranes and leads to enhanced delivery of granzymes into target cells.[5] Another pathway to cytotoxicity, which is utilized to a lesser extent than GZMB- and perforin-mediated killing, is through the binding of Fas ligand (FasL) on CD8⁺ T cells to Fas on target cells, which induces programmed cell death of the target cell.[18]

As with CD4⁺ T cells, there are several subtypes of CD8⁺ T cells. One subtype, identified as either "cytotoxic T lymphocytes" (CTLs), or T_{C1} cells, produces high levels of cytolytic cytokines such as GZMB, IFNγ and TNF. In a cancer setting, high levels of intratumoral T_{C1} are associated with positive therapeutic outcomes. Other CD8⁺ subgroups, while still cytolytic (albeit to a lesser extent than T_{C1} cells), produce a variety of different cytokines and play distinct roles in the immune responseT_{C2} cells produce IL-4 and -5, and T_{C9} cells produce IL-9; these subsets are drivers of allergic responses. T_{C17} and T_{C22} cells produce IL-17 and IL-22, respectively; their clinical significance has not been fully elucidated. There is a subset of follicular CD8+ T cells (T_{FCS}) which are thought to enhance B cell responses. Finally, two regulatory CD8+ T cell ($CD8^+T_{REG}$) subtypes have been identified, one of which has been found to be present at high levels in people with autoimmune disease, and another which appears to play a role in suppressing graft-versus-host disease.[19] CD8⁺ T cell subsets are less well-defined than CD4⁺ T cell subsets, and some of the evidence supporting their various functions has so far only been found *in vitro*.

1.2.5 Memory phenotype

Prior to activation, T cells exist in a naïve state (T_N); activation induces differentiation into a variety of memory subsets. Generally, memory phenotype is defined depending on the expression or non-expression of surface markers associated with different traits, such as the capacity to home to specific tissues.[20] Four memory phenotypes in particular are discussed in this thesis – T_N, central memory (T_{CM}), effector memory (T_{EM}), and effector memory re-expressing CD45RA (terminally differentiated, T_{EMRA}). Importantly, there are several other T cell memory subsets that are not explicitly discussed in the work shown here but are nevertheless important parts of the immune landscape, including the stem cell memory (T_{SCM}) and tissue resident memory (T_{RM}) subsets.[13]

In recent years, T cell naming conventions have been scrutinized – subsets names are used in a fashion that is too nebulous to be practically useful. This is problematic as a shared language and shared understanding of what is meant when referring to a given subset is essential for interpreting results and building on the work and findings of others. Further, as T cell research has advanced, results are increasingly indicating that T cell subsets are less black and white and are more likely to occupy a gray zone throughout their differentiation, expressing surface molecules on a scale rather than according to a binary.[13] This being said, there are still widely used methods of identifying T cell differentiation state which, while they might not be reflective of the whole truth, are useful for a basic mapping of which memory compartments are present in a T cell population. In this thesis, we use the C-C chemokine receptor 7 (CCR7) and CD45RA, a tyrosine phosphatase that regulates T cell activation,[5] to define T_N, T_{CM}, T_{EM}, and T_{EMRA}. Characteristics of these cells and how we define them here are outlined in Table 2.[5,13]

Subset	Description	Defined here as	Also defined as
T_N	T cells that have exited the thymus and have not encountered cognate antigen. Undifferentiated.	$CCR7^+CD45RA^+$	$CD45RO^-$; $CD95^-$; $CD27^+$: $CD28^+$; $CD127^+$; $CD11a^{lo}$; $CD122^-$

T_{CM}	T cells that express lymph node-homing receptors that allow them to recirculate between lymph and blood; reside in secondary lymphoid tissues. Can differentiate further into T_{EM}.	$CCR7^{+}CD45RA^{-}$	$CD62L^{+}$; $CD27^{+}$; $CX3CR1^{+}$; $CD45RO^{+}$
T_{EM}	T cells that lack lymph node-homing receptors and cannot recirculate through lymph nodes; found in peripheral blood and tissues.	$CCR7^{-}CD45RA^{-}$	$CD62L^{-}$
T_{EMRA}	Effector memory T cells that express CD45RA. Cytolytic; express GZMB and perforin. Terminally differentiated.	$CCR7^{-}CD45RA^{+}$	$CD27^{-}$; $CX3CR1^{hi}$; $CD57^{+}$

Table 2. Memory T cell subsets.

1.2.6 T cell metabolism

Cellular metabolism refers to the reactions within a cell that provide it with energy, or adenosine triphosphate (ATP).[21] It is an endlessly complex field, but what is important to know for the purposes of the work described here is that T cells upregulate or downregulate metabolic pathways depending on nutrient availability, differentiation state, and subtype. Metabolic plasticity, or the ability to switch between metabolic pathways depending on need and/or nutrient availability, is key for T cells' ability to mount adequate responses. Presence or absence of nutrients in T cells' environment regulates their ability to differentiate, and in a nutrient-poor environment, even in the presence of activation signals, without fuel, they cannot function.[22] Two particularly important pathways are glycolysis and oxidative phosphorylation (OXPHOS).

Glycolysis is an oxygen-independent pathway in which extracellular glucose is taken up and metabolized in the cytosol; one molecule of glucose yields two molecules of ATP. Activated T cells will sometimes use glycolysis even in the presence of adequate oxygen to fuel OXPHOS; this is referred to as "aerobic glycolysis", and takes place when rapid proliferation occurs. Originally thought to be specific to cancer cells, it is now known that highly proliferative cells, like T cells, use this pathway instead of the more energy-efficient OXPHOS because glycolysis produces large amounts of biosynthetic intermediates that can be shuttled into other metabolic pathways and anabolic processes to support activation and clonal expansion.[23,24]

OXPHOS is an aerobic metabolic pathway that takes place in the mitochondria, and refers to the process by which the electron transport chain (ETC)which is composed of a series of protein complexes (complex I-IV) in the inner mitochondrial membrane, and the ATP synthase complex (complex V in the inner mitochondrial membrane) generate ATP. OXPHOS yields up to 18 times as much ATP as glycolysis,[25] and is utilized to a high degree by naïve T cells, which only need a basal energy production to maintain homeostasis.[26]

Reactive oxygen species (ROS), including O_2^-, hydrogen peroxide, and hydroxyl radicals are produced during aerobic metabolism.[27] Healthy cells maintain redox homeostasis, or a balance of cellular antioxidants and ROS. ROS are vital for intracellular signaling and multiple metabolic processes which in turn impact proliferation and differentiation. When redox homeostasis is disrupted and the balance skews towards ROS, it is referred to as oxidative stress, and in this context, ROS are associated with DNA damage and other detrimental effects.[28]

1.2.7 Transcription factors

Cellular metabolism is regulated by several important transcription factors. Two of these are the mechanistic target of rapamycin (mTOR) and hypoxia-inducible factor 1 (HIF-1).

mTOR is a master regulator of cellular metabolism. It is a serine/threonine kinase present in T cells and other cells which acts as a catalytic subunit for mTOR complexes 1 (mTORC1) and 2 (mTORC2).[29,30] mTORC1 and mTORC2 contribute to regulation of cell metabolism in different ways, for instance through phosphorylation of metabolic enzymes.[29] mTORC1 upregulation leads to the promotion of glycolytic metabolism over OXPHOS, in part by increasing translation of HIF-1α.[30] Interestingly, in dividing cells, mTORC1 inheritance is

asymmetric, which has profound metabolic and impacts on the fate of each respective daughter cell.[24]

HIF-1 is a transcription factor composed of two subunits – HIF-1β, which is constitutively expressed, and HIF-1α, which is inducible. HIF-1α is stabilized at low oxygen tensions and degraded at higher levels;[22] in T cells, TCR activation also leads to stabilization of HIF-1α even at normal physiological oxygen levels [31]. HIF-1α is arguably the most important transcription factor for adaptation to hypoxia, partly due to its role in upregulating the glucose transporter GLUT1, which is used to import extracellular glucose.[32] Palazón and colleagues used a conditional knockout model to demonstrate that T cells lacking HIF-1α were unable to control tumors, likely due to faulty expression of genes necessary for glycolysis in combination with an observed significant decrease in effector cytokine production by these cells.[31]

1.3 Immune surveillance and cancer immune evasion

Cells of the innate and adaptive immune system cooperate to monitor the body for damaged and diseased cells and extracellular pathogens; this is referred to as "immune surveillance".[33] Through this process, the immune system is able to identify and destroy early-stage malignancies. The balance between recognizing tumor cells as foreign while not tipping into autoreactivity is referred to as the cancer-immunity cycle.[34] Selective cytotoxicity against malignant-transformed cells is thought to create a kind of evolutionary pressure that may lead to cancer cells "learning" how to avoid detection by the immune system, either through downregulating antigen presentation or through accumulation of mutations that favor survival.[35]

Cancerous cells communicate with their environment from very early stages of disease development, recruiting cells that contribute to tumor formation, such as tumor-associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs).[36] The tumor's continued development generates an immunosuppressive TME, containing T_{REG} cells and myeloid-derived suppressor cells (MDSCs), which can produce detrimental levels of ROS.[35] Tumor cells themselves can express inhibitory signals such as the immune checkpoint ligand programmed death-ligand 1 (PD-L1), which binds to programmed death-1 (PD-1) on T cells, causing T cell dysfunction.[37] Perturbed intratumoral angiogenesis leads to inadequate supply of nutrients and oxygen for immune cell function within the tumor.[38] Importantly, many established tumors remain at least partly

immunogenic, and despite this fact they are unable to be cleared by the immune system. This underscores the significance of the early recruitment of immunosuppressive cells and establishment of an unfavorable environment in immune escape by neoplasms.[39]

1.4 Immunotherapy

Over the last several years multiple kinds of immunotherapy – treatments that harness the immune system to fight disease- have become increasingly prevalent, opening the door to treating and curing heretofore untreatable diseases. Several treatments fall under the umbrella of immunotherapy; two of the most notable are the immune checkpoint inhibitors (ICIs) and adoptive cell therapy (ACT).

1.4.1 Immune checkpoint inhibitors

The immune checkpoints are several receptors that function to suppress T cell responses. They are vital for normal immune function, where they restrain immune responses, but can be detrimental in the context of cancer, where their expression can suppress T cell activity against cancer.[40] Some important immune checkpoint molecules are cytotoxic T lymphocyte-associated protein 4 (CTLA-4), programmed death-1 (PD-1), and its ligands, programmed death ligand 1 and 2 (PD-L1/2), lymphocyte activation gene-3 (LAG-3), and T cell immunoglobulin and mucin-domain containing-3 (TIM-3).

CTLA competes with CD28 for binding to CD80/86 on tumor cells, and has a much higher binding affinity for these receptors, preventing T cell co-simulation and instead leading to restrained T cell activation, anergy, and finally apoptosis.[18] The association of PD-1, which is expressed on T cells, and PD-L1/PD-L2, which are expressed on tumor cells, interrupts co-stimulation through CD28, which inhibits TCR signal transduction, ultimately leading to the inhibition of several vital functions of T cells, including cytokine production. Further, engagement of PD-1 initiates a metabolic switch from glycolysis to fatty acid oxidation, leading to a decrease in production of biological intermediates and an increase in frequency of ROS,[18] disrupting oxidative balance within the cell.

ICIs are monoclonal antibodies that bind to and block the function of immune checkpoints. The best-studied and most widely used checkpoint inhibitors block CTLA-4 and PD-1/PD-L1/2;[40] most recently, a LAG-3 inhibitor was added to the treatment arsenal. ICI response rates are variable, largely depending on disease type;[41] one especially notable result was achieved in the treatment of

patients with mismatch repair-deficient, locally advanced colorectal cancer with an anti-PD1 checkpoint inhibitor, leading to recurrence-free survival at two years in 92% of patients.[42] Anti-PD-1 inhibitors have also been efficacious against metastatic melanoma, and have been demonstrated to induce complete responses in a matter of weeks.[18]

1.4.2 Adoptive cell therapy

In contrast to ICI, which involves treating a patient with a monoclonal antibody to stimulate his or her own immune system, ACT involves harvesting immune cells, modifying them *ex vivo*, and then infusing them into a patient. ACT can be broken down into three groups: tumor-infiltrating lymphocyte (TIL) therapy, T cell receptor-engineered T cell (TCR-T) therapy, and chimeric antigen receptor (CAR) T cells.

1.4.2.1 TILs

TILs are T lymphocytes harvested from resected tumors which are expanded *ex vivo* in the presence of fragments of tumor, and then reinfused into the patient.[43] TILs have mostly been studied in the context of advanced melanoma and extremely impressive results have been reported.[44] TILs are under investigation for use in non-cutaneous solid tumors including ovarian cancer.[45] One key challenge of TIL therapy is that, in order for it to be feasible, the tumor in question has to contain tumor-specific T cells, and far from all solid tumors do.[2]

1.4.2.2 TCR-T cells

TCR T cells are T cells engineered *ex vivo* to express TCRs that specifically target tumor antigen.[46] This modality is MHC-restricted[2] and has the advantage of being able to recognize both surface antigens and intracellular antigens. TCR-T cells have been evaluated in clinical trials against a variety of antigens expressed on a range of diseases, including melanoma, sarcoma, and gastrointestinal, cervical, lung, and liver cancers. So far results have been modest, due to antigen heterogeneity and the inhospitable TME.[44]

1.5 CAR T cell therapy

Chimeric antigen receptors combine the antigen specificity of an antibody with the internal signaling mechanism and cytotoxic capacity of a T cell. CARs, unlike TCRs, are not MHC-restricted, and can thus be directed to target any selected surface marker.[47] There are currently six commercially available CAR T cell

products approved by the European Medicines Agency (EMA), outlined in Table 2.[48] These CAR T cell products have regulatory approval for the treatment of the hematological malignancies acute lymphocytic leukemia (ALL), diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL), follicular lymphoma grade 3B (FL3B), high-grade B cell lymphoma (HGBL), primary mediastinal B cell lymphoma (PMBCL), mantle cell lymphoma (MCL), chronic lymphocytic leukemia (CLL), and multiple myeloma (MM). CAR T cells are, with few exceptions, only approved for use in relapsed or refractory diseases after 2 lines of other types of therapy have been tried.[48]

Product	CAR construct	Clinical indication
Kymriah (tisagenlecleucel, tisa-cel)	CD19 – 4-1BB – CD3ζ	ALL; DLBCL; FL
Yescarta (axicabtagene ciloleucel, axi-cel)	CD19 – CD28 – CD3ζ	DLBCL; FL; HGBL; PMBCL
Tecartus (brexucabtagen autoleucel, brexu-cel)	CD19 – CD28 – CD3ζ	ALL; MCL
Breyanzi (lisocabtagene maraleucel, liso-cel)	CD19 – CD28 – CD3ζ	CLL; DLBCL; FL3B; PBMCL
Abecma (idecabtagene vicleucel, ide-cel)	BCMA – 4-1BB – CD3ζ	MM
Carvykti (ciltacabtagene autoleucel, cilta-cel)	BCMA – 4-1BB – CD3ζ	MM

Table 3. EMA-approved CAR T cell therapies.

1.5.1 CAR generations

To date, there are five widely accepted CAR generations. First-generation CARs are composed of an antigen-binding domain (shown in Figure 3[49]), composed of a single-chain variable fragment (scFv) derived from the heavy and light chain variable regions of an antibody (V_H and V_L, respectively) bound by a linker,

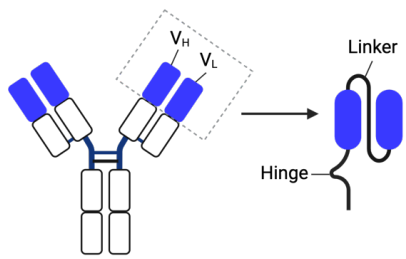

Figure 3. Chimeric antigen receptor scFv. Adapted from Flugel et al, Nat Rev Clin Oncol 2023.

connected via a hinge domain to a transmembrane domain and an intracellular CD3ζ chain. Of the CD3 chains, CD3ζ was chosen in particular due to its known involvement in mediating various activation programs triggered by the TCR binding to its cognate antigen;[50] interestingly, a recent study demonstrated that substituting the CD3ζ with other CD3-derived cytoplasmic tails – CD3δ, -ε, or -γ - conferred distinct functional characteristics on the CAR T cells compared to the classical CD3ζ tail; in particular, CARs containing the CD3δ chain showed improved killing and lower expression of PD-1.[10] While first-generation CAR T cells were able to target antigen-expressing cells *in vitro*,[51] they functioned poorly in patients: ovarian cancer-directed first-generation CAR T cells showed a lack of persistence and poor trafficking to the tumor.[52]

It was subsequently recognized that an activation signal through the CD3ζ chain alone may not be sufficient to elicit a T cell response. Since native T cells require both TCR engagement and a second signal by engagement of a co-stimulatory receptor for activation, it was reasoned that this missing second signal could be what was holding CAR T cells back from optimal function.[53] This was the advent of the second-generation CAR T cell, which added an intracellular co-stimulatory domain to the CAR construct, between the transmembrane and intracellular signaling domains. In a murine model of ALL, second-generation CAR T cells demonstrated enhanced antitumor activity even in the absence of exogenous co-stimulation,[54] and in 2011, the first report of complete remission in subjects with r/r ALL in response to treatment with 1928ζ CAR T cells was published.[55]

If one co-stimulatory domain is better than none, it follows that two must be even better. Thus, the third-generation CAR containing two co-stimulatory domains was conceived.[56] Carpenito and colleagues demonstrated *in vivo* that combining 4-1BB and CD28 co-stimulatory domains had improved antitumor effects and persistence compared to either co-stimulatory domain alone.[57] Enblad and colleagues performed a first-in-man study with third-generation CAR against r/r B cell lymphoma and leukemia and found that while clinical outcomes were similar to second-generation CAR, third-generation constructs appeared to have extended activation subsequent to engagement with cognate antigen.[58]

Fourth-generation CARs, also known as "armored CARs" or T cells redirected for universal cytokine-mediated killing (TRUCKs), are CARs engineered

to produce cytokines such as IL-2 and -7 in order to support proliferation, survival, and function of CAR T cells,[59] or IL-12, in order to support polarization into a favorable T_H1 phenotype.[11] Zhao and colleagues demonstrated that CAR T cells engineered to produce the anti-inflammatory cytokine IL-10 outperformed conventional CAR T cells in an *in vivo* model of B cell lymphoma, promoting CAR T cell proliferation and enhancing their effector function, in part by sustaining their mitochondrial fitness.[60]

Fifth-generation CARs take advantage of the Janus kinase (Jak) and signal transducer and activator of transcription (STAT) pathway. In T cells, the intracellular Jak-STAT mechanism is associated to surface cytokine receptors. When cytokines, such as interferons or interleukins, bind to these receptors, Jak mediates phosphorylation of the intracellular receptor tail and recruits one or more STATs, which participate in signal transduction. STATs 3 and 5 are of particular importance for T cell metabolism.[61,62] Fifth-generation CARs contain a modified IL-2 receptor β chain (IL2Rβ) and STAT3-binding domain between a CD28 co-stimulatory domain and CD3ζ. The addition of IL2Rβ activates JAK kinase and STAT3 and -5 signaling pathways, leading to improved *in vivo* persistence and cytolytic activity in both hematological malignancies and solid tumors.[63]

An overview of the five generations of CAR constructs is shown in Figure 4.[64]

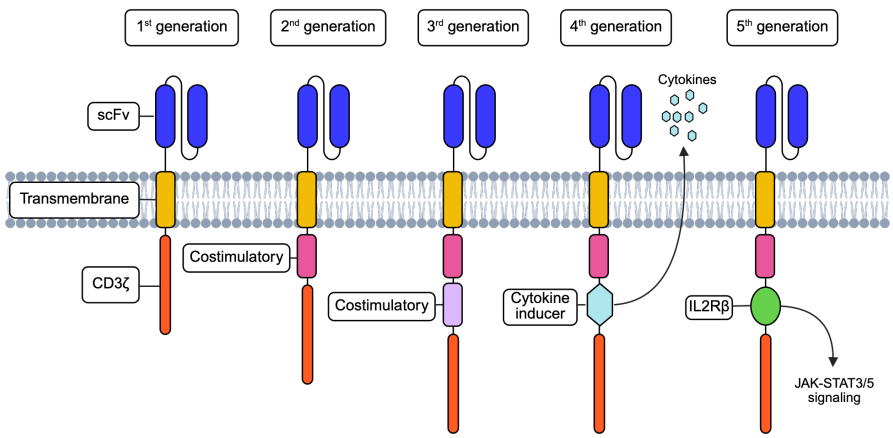

Figure 4. CAR generations. Adapted from Amorós-Pérez et al MDPI Cancers 2024..

1.5.2 Co-stimulation

The two most commonly utilized co-stimulatory domains are CD28 and 4-1BB (CD137). CD28 and 4-1BB activate different signaling pathways and are, in CAR T cells, associated with the development of distinct phenotypic and functional profiles.

CD28 is a member of the immunoglobulin superfamily. It is endogenously expressed on the surface of naïve T cells, and has a pivotal role in T cell activation. As discussed previously, CD28 is engaged by CD80/86 on the surface of APCs. CD28 ligation recruits phosphatidylinositol 3-kinase (PI3K), which in turn activates Akt (protein kinase B)[18] – this has several consequences, including activation of mTOR, upregulation of IL-2 production and a spike in glucose uptake.[32,65] CD28 co-stimulated CAR T cells are highly glycolytic and have powerful effector functions, reflecting endogenous qualities associated with CD28.[66]

4-1BB is a member of the tumor necrosis factor receptor (TNFR) superfamily, and is endogenously expressed on the surface of activated $CD4^+$ and $CD8^+$ T cells. It binds to its ligand 4-1BBL, which is expressed on APCs and activated T cells. Signaling through 4-1BB recruits TNFR-associated-factors (TRAFs) 1 and 2, which form complexes that induce signaling through canonical and non-canonical nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), which leads to enhanced cytokine secretion and proliferation.[67] 4-1BB co-stimulated CAR T cells have a less dramatic initial effector response compared to CD28 co-stimulated CARs, but tend to persist longer, likely related to the fact that 4-1BB ligation promotes mitochondrial biogenesis, thereby supporting oxidative metabolism. Further, 4-1BB co-stimulation promotes the enrichment of a central memory phenotype. CD28 co-stimulation, in contrast, seems to promote an effector memory phenotype.[32,68]

1.5.3 Modifying classic CARs

Apart from the five CAR generations described previously, there are several dozen other modifications to "classic" CAR structures that have yielded some very exciting results.

The 1xx CAR, which is utilized in this thesis, has a modified CD3ζ chain; the two most distal ITAMs are mutated and nonfunctional, calibrating intracellular

activation potential. In an *in vivo* model of ALL, second-generation anti-CD19 1xx CAR T cells outperformed standard CAR T cells, rapidly eradicating tumors and eliciting long-term remissions.[69] 1xx CAR structure is shown in Figure 5.

The location of CAR integration in the T cell genome has also been evaluated as a factor contributing to CAR T cell responses. CAR T cells are generally transduced using retro- or lentiviral vectors, leading to random integration of the CAR vector into the genome. Using CRISPR/Cas9 to target the CAR to the T cell α constant locus, investigators were able to control CAR expression, which was shown to significantly enhance CAR T cell function.[70]

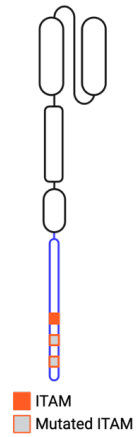

Figure 5. 1xx CAR

CAR structural organization can also be adjusted, with favorable outcomes: third-generation CAR T cells attempt to harness the features of distinct co-stimulatory domains by placing them end-to-end in a linear fashion, but it was noted that some of the functions of the most distal co-stimulatory domain were being lost due to positioning far from the cell membrane. This loss could be abrogated by the arrangement of the co-stimulatory domains parallel to each other; the parallel CAR (pCAR), as it is called, contains a second-generation CAR T cell fused at the co-stimulatory receptor to a parallel chimeric costimulatory receptor expressing a second co-stimulatory domain. In an *in vitro* setting, the pCAR achieved enhanced tumor control compared to standard second- and third-generation CAR constructs.[71]

In the vein of fourth-generation CAR T cells, the CAR(NAP) construct expresses neutrophil-activating protein (NAP) derived from *Helicobacter pylori*, which triggers bystander T cell responses. This was shown to lead to the reinvigoration of a "hot" pro-inflammatory environment intratumorally.[72]

1.5.4 CAR targets

All commercially available CARs target either CD19 or B cell maturation agent (BCMA), which are cell-surface antigens expressed on B cells and B cell malignancies. These antigens are great examples of optimal CAR targets: they are highly expressed on target tissues, and their expression level on normal tissues is restricted to B cells, an "expendable" tissue. Off-tumor expression is important to consider in the selection of CAR targets; B cell aplasia is survivable and an expected side effect of anti-CD19/BCMA CAR T cells,[73,74] but on-target off-tumor

toxicity can be lethal if CARs are targeted against antigens that are expressed on vital tissues

Solid tumors have proven significantly trickier to target than hematological malignancies. There are several CAR targets for solid tumor disease at various stages of investigation, with variable results. One CAR target that has been widely investigated is mesothelin (MSLN), a surface glycoprotein overexpressed on many different tumor types, including ovarian, lung, and pancreatic cancers, but is also physiologically expressed – albeit at low levels – on normal mesothelial cells present in the pleura, peritoneum, and pericardium.[75] Several CAR T cell constructs targeting MSLN exist and are under investigation. *In vitro,* anti-MSLN CAR T cells have been able to efficiently target and eliminate MSLN-expression tumor cells, and they are able to control tumor growth *in vivo* in different disease models; in clinical trials, anti-MSLN CAR T cells have been widely investigated against a range of MSLN-expressing solid tumors, with modest clinical responses at best. One important finding of these studies has been that anti-MSLN CAR T cells appear to generally be safe for use,[76] though a high-affinity anti-MSLN CAR was recently implicated in the deaths of two patients through on-target off-tumor toxicity.[77]

1.5.5 Cellular substrate

All commercially available CARs use T cells as a substrate. As discussed, T cells have innate killing capabilities and an ability to establish immune memory; they are also capable of homing to tissues, qualities which made them the natural choices as cell substrate for the earliest CAR T cells.[50] Following the success of CAR T cells, interest arose in evaluating other cell types as substrates.

$\gamma\delta$ T cells possess several potentially favorable endogenous qualities. They are not MHC-restricted, possess innate-like killing capabilities, and have been demonstrated to be able to infiltrate tumors; in fact, their presence in tumors has been tied to favorable outcomes in a variety of diseases. In the clinic, $\gamma\delta$ CAR T cells have so far demonstrated acceptable safety profiles and promote antigen-specific antitumor cytotoxicity; results for B cell lymphomas are promising.[78] $\gamma\delta$ CAR T cells are also being investigated for use in a number of solid tumor diseases.[79]

CAR NK cells have also been investigated; like $\gamma\delta$ T cells, NK cells are not MHC-restricted, and are able to mediate cytotoxicity both through their CAR but also through endogenous receptor expression, including CD16. CAR NK cells

are also expected to be safer than CAR T cells;ne common side effect of CAR T cell therapy is cytokine release syndrome (CRS), which can be life-threatening and often requires intensive care; CAR NK cells, which have a different cytokine secretion profile than CAR T cells, do not appear to elicit CRS in a clinical setting.[80] Early results demonstrate that CAR NK cells have a highly favorable safety profile in B cell lymphoma, and otherwise have limited but modestly successful clinical responses. Solid tumor data are limited for this modality.[81]

Interest arose in CAR macrophages in part to overcome the issue of cellular trafficking into the tumor; macrophages are abundant in several solid tumor diseases, in part because they are recruited by the tumors themselves. TAMs, while immunosuppressive, are still phagocytotic, and in an *in vitro* setting, expression of a CAR redirects them to specifically phagocytose cancer cells.[81]

1.5.6 Favorable T cell identities

Memory phenotype of infusion product seems to be key for successful CAR T cell therapy in hematological malignancies. CAR T cells derived from T_N and T_{SCM} have been demonstrated to perform better *in vivo* than more differentiated cells.[82,83] T_{CM} also display superior cytotoxicity compared to more differentiated phenotypes.[84]

The ratio of $CD4^+$:$CD8^+$ is also significant; physiologically, $CD4^+$ and $CD8^+$ CAR T cells operate synergistically, with $CD4^+$ CAR T cells producing significantly more cytokines than $CD8^+$, and while $CD4^+$ can mediate cytotoxicity, $CD8^+$ CAR T cells are significantly better at it. In one study of "leftover" infusion products taken from axi-cel (1928ζ), tisa-cel (19BBζ), or brexu-cel (1928ζ) CAR T cells generated for use in patients, investigators found that a low CAR $CD4^+$:$CD8^+$ ratio at the time of infusion was predictive of positive response rate at three and six months following treatment.[85] In a ten-year follow-up two patients with CLL treated with anti-CD19 CAR T cells who achieved complete, durable remission, investigators found populations of functional, exclusively $CD4^+$ CAR T cells present in both individuals, suggesting that continued remission might be mediated by this compartment.[86]

Cellular metabolism and especially metabolic plasticity are vital for CAR T cell function. High mitochondrial mass is connected to the ability to switch between OXPHOS and glycolysis. High mitochondrial membrane potential, conversely, is associated with effector memory T cells and is reflective of a glycolytic state and high levels of ROS production, which can result in exhaustion;

low mitochondrial membrane potential, is associated with enhanced persistence.[87]

1.5.7 CAR T cell manufacturing

CAR T cells are generally produced according to the same basic principles (outlined in Figure 5): a patient undergoes leukapheresis, and T cells are isolated, activated, transduced, and expanded before being reinfused into the patient.[39]

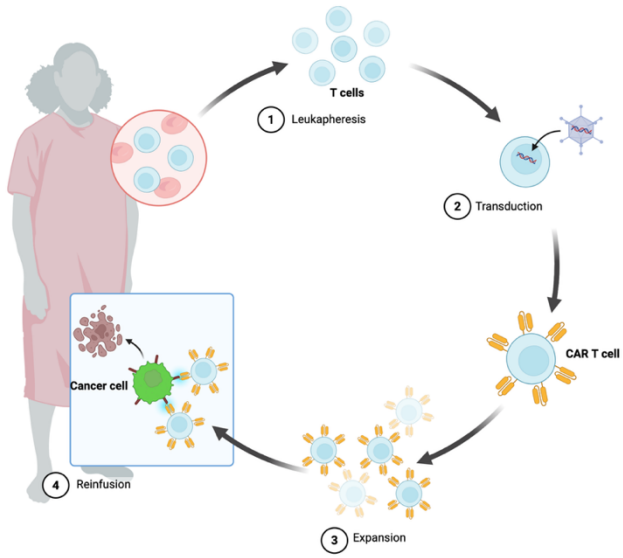

Figure 6. CAR T cell production overview

Before CAR T cell infusion, patients usually undergo lymphodepleting chemotherapy, which promotes CAR T cell engraftment.[88] Because of lengthy production times, vein-to-vein time may be several weeks.[89] In order to avoid disease progression between leukapheresis and the initiation of lymphodepleting chemotherapy, bridging therapy may be used (usually chemotherapy; other options are immunotherapy and/or targeted therapy).[90]

1.5.8 Impact of supraphysiological oxygen on cells *in vitro*

In vitro cell culture is typically performed at atmospheric oxygen levels, ≅21% O_2. This is a level that is never encountered by cells *in situ*; inhaled oxygen passes through several physiological filters as it makes its way from the lungs to the bloodstream and finally into the tissues. In the human body the highest oxygen levels, ≅14%, are found in lungs and arterial blood and decrease from there, with the lowest reported levels being ≅1% in mitochondria[91] (reported average oxygen

levels vary; 1-14% is a commonly accepted physiological range, but some investigators cite wider ranges, from 0-19%[92]).

Oxygen availability impacts absolutely everything in a biological system. Cellular metabolism, angiogenesis, proliferation and more all depend on oxygen tension.[92] In T cells, exposure to atmospheric (i.e. supraphysiological) oxygen levels leads to significantly augmented proliferation, which is a favorable characteristic in the context of adoptive cell therapies, where a minimum cell number must be achieved in order to treat a patient. The other side of this coin is that upregulated aerobic metabolism leads to the production of ROS, and oxidative imbalance.[93]

The potential drawbacks of culturing primary T cells at supraphysiological oxygen levels are well known. Prolonged exposure to atmospheric oxygen levels changes transcriptional programming, impacts mitochondrial fitness,[91] and throws off the intracellular oxidative balance, causing oxidative stress.[93] However, investigators that make these observations often include the caveat that what this practically means for applications like *in vitro* experiments is unclear and likely not significant enough to warrant a change in conventional culture methods in favor of performing cell culture at more physiologically relevant oxygen levels.[91] Optimizing oxygen availability in culture conditions is likely more important for processes like CAR T cell production, where T cells from heavily pretreated patients are exposed to ambient oxygen levels, sometimes for weeks at a time, and where oxidative stress and metabolic remodeling may be highly significant for the function of the resultant CAR T cell product.

1.5.9 CAR T cell interplay with the non-CAR T cell compartment

Several investigators have sought to establish correlations between the intratumoral immune landscape and its response to CAR T cell infusion, including positive and negative correlates between the native T cell population and clinical outcomes. Clinical outcomes have shown that a highly active intratumoral immune environment before and after CAR T cell infusion, including the presence of a cytotoxic, highly proliferative non-CAR (bystander) T cell population,[94] along with high expression of cytokines and chemokines are positive predictors for response.[95]

High frequency of T_{REG} cells was associated both with a blunted response to CAR T cell therapy as well as a lesser degree of CAR T cell-related

side effects, including neurological side effects.[94,95] Low frequency of T_{REG} cells was identified as predictive of durable response. Bystander memory phenotype after T cell infusion has also been linked to clinical responses; a highly cytotoxic effector memory $CD4^+/CD8^+$ T cell compartment was predictive of durable response, while minimally cytotoxic T_N/early memory T cells were shown to be associated with early relapse.[94]

Lymphodepletion and CAR T cell infusion quickly modulate the immune landscape within a tumor; in 2025, Frede and colleagues identified a $CD8^+$ T cell population enriched in highly differentiated effector cells that emerged in response to these interventions and was predictive of poor treatment response, also noting that $CD8^+$ T cells with a memory-like gene signature were associated with long-term responses. Importantly, CAR T cell treatment seems to impact the T cell compartment even after CAR T cells are no longer physically present.[96]

1.5.10 CAR T cells to date

CAR T cells have revolutionized the treatment of B cell malignancies, demonstrating significantly improved clinical outcomes compared to standard therapies. However, outcomes for patients treated with commercially approved CAR T cells are inconsistent, and in fact most patients either respond only partially or experience disease recurrence after treatment.[4] The reasons for unsuccessful CAR therapy are incompletely understood, and the hurdles to therapy we have identified have so far proven exceedingly difficult to clear. Despite encouraging *in vitro* and *in vivo* results and occasional favorable Phase I trial reports, there are still no available CAR T cell therapies for solid tumor disease. A deeper understanding of CAR T cell intratumoral kinetics, along with continued investigation into approaches to optimize CAR T cell therapies for inhospitable disease environments is needed.

1.5.11 Why CAR T cells fail

There are several reasons why CAR T cells may fail to elicit complete or durable responses. Three main factors are discussed here: malignancies' ability to evade immune destruction, physical barriers to tumor infiltration, and the immunosuppressive tumor microenvironment.

1.5.11.1 Avoiding immune destruction

Tumor cells are able to avoid detection by immune cells using several strategies, including by downregulating antigen presenting mechanisms. Trogocytosis, a

physiological process that involves horizontal transfer of proteins between cells, can lead to tumor antigen loss.[97] Trogocytosis frequently occurs in conjunction with binding at the immunological synapse, and practically means that T cells, including CAR T cells, can "pick up" surface molecules, including antigen, from the tumors they target. This means that while the target effectively becomes invisible to the CAR T cell, CAR T cells themselves express the target antigen, putting them at risk of being targeted by other CAR T cells through fratricide killing.[98] High affinity between a CAR and its target appears to contribute to loss of antigen in B cell malignancies; this can be countered by reducing CAR affinity;[99,100] importantly, in a model of MSLN-expressing cancer, Yang and colleagues demonstrated that affinity-tuned anti-MSLN CAR T cells mediated robust on-tumor cytotoxicity while reducing off-tumor toxicity.[75]

1.5.11.2 Physical barriers

From their earliest stages, tumor cells are able to generate their own noxious microcosms, closed off to immune regulation by extracellular matrix, MDSCs, TAMs, CAFs, and more. Chemotherapy is widely used in the treatment of solid and liquid malignancies, and in the context of CAR T cell therapy it is frequently used as bridging therapy and/or as lymphodepletion prior to CAR T cell reinfusion, which improves CAR T cell engraftment.[101] Standard lymphodepletion chemotherapy before commercial CAR T cells is a combination of fludarabine (Flu), a purine analogue, and cyclophosphamide (Cy), an alkylating agent.[88,102] In the context of solid tumor-directed CAR T cells, preconditioning with various chemotherapeutic agents has been demonstrated *in vitro* and *in vivo* to reduce the number of intratumoral T_{REG}s or inhibit their function, downregulate the expression of immune checkpoint ligands, promote CAR T cell infiltration, polarize macrophages from an M2 inhibitory to an M1 inflammatory state, and enhance T cell trafficking to tumors.[101]

One important consideration in developing lymphodepletion protocols for use in conjunction with CAR therapy is that many antineoplastic drugs exert potent dose-dependent immunosuppressive effects and may for that reason not be optimal for combining with ACT. The effect of antineoplastic drugs on anti-solid tumor CAR T cells is not fully understood; while it is known that these drugs impact tumors and the hostile TME in multiple ways, including by repressing immunosuppressing cells and disrupting stromal components, more research is needed to evaluate drug combinations and dosage rates in order to disrupt tumors without damaging CAR T cells.[101,103] In the work shown here, we evaluate the

potential of the combining Flu and the alkylating agent Treosulfan (Treo)[104] with CAR T cells for use in a solid tumor context.

1.5.11.3 Hypoxia in the context of the TME

The TME is an incredibly hostile environment. Dysregulated cell expansion contributes to aberrant vascularization and results in low nutrient and oxygen availability, along with extracellular acidification.[105] In this thesis the hypoxic nature of the TME has been an area of specific focus; as discussed, physiological oxygen levels are usually reported to be between 1-14%; in a tumor, oxygen can be <2%, even as low as 0% (anoxia). Oxygen levels this low can lead to tumor escape through the downregulation of MHC class I[106] or the reduction of cell-surface antigen expression.[107] Intratumoral hypoxia can lead to recruitment of immunosuppressive cells including MDSCs, TAMs and T_{REG}s.[108,109] Inadequate oxygen availability is well-known to lead to T cell dysfunction.[106] Though not exhaustively studied or completely understood, there have been several studies performed with the aim of understanding the impact of pathologically low oxygen on T cells and CAR T cells by activating and culturing these cells with extremely restricted oxygen availability, with varying results.

One study examined intratumoral kinetics and function of $CD8^+$ T cells activated and cultured in 1% O_2, and found that while hypoxia-adapted T cells were not able to better penetrate poorly vascularized areas better compared to normal T cells, they did demonstrate significantly enhanced tumor control and prolonged survival *in vivo*.[110] In contrast, another study found that hypoxic CAR T cells, which were not activated in hypoxia but rather transferred into 1% O_2 on day five of expansion, displayed significantly impaired proliferation and cytotoxicity *in vitro*. Hypoxia also led to an accumulation of T_{CM} cells; the authors interpreted this as an impaired differentiation into T_{EM}, albeit without excluding the possibility that hypoxia in fact led to an increased differentiation from T_N into T_{CM} and promoted the maintenance of this compartment.[111] Time-limited exposure of $CD8^+$ T cells to 1% O_2 during activation led to increased mitochondrial metabolism and increased tumor killing *in vivo*, compared to prolonged exposure to hypoxia during activation, which led to impaired T cell activation and subsequent dysfunction.[112] Most recently, a study evaluating different CAR T cell expansion platforms (i.e. culture bags, plates etc.) found that low dissolved oxygen in cell cultures led to the enrichment of T_N cells which, as discussed, is associated with clinical benefits.[113]

1.6 Disease models used in this thesis

1.6.1 Ovarian cancer

Ovarian cancer has a dismal prognosis; it is the deadliest gynecological malignancy, which is primarily thought to be due to late detection.[114] 90% of ovarian cancers are of epithelial origin (epithelial ovarian cancer, EOC),[115] and we worked exclusively with models of EOC in this thesis. In Sweden, the standard of care for EOC at time of discovery is surgery, with neoadjuvant or adjuvant chemotherapy depending on disease stage. In the case of disease recurrence, clinical presentation of disease dictates management; one or several of surgery, chemotherapy (different modalities), the vascular endothelial growth factor (VEGF) inhibitor bevacizumab, or poly-ADP-ribose polymerase (PARP) inhibitors, which inhibit single-strand DNA breakage repair, are common approaches to manage recurrent disease.[114] Development of new therapies is of paramount importance to improve patient survival.

1.6.2 CLL (CD19-expressing disease in hypoxia)

As discussed, while remission is induced in many patients with hematological malignancies treated with CAR T cells, many do not respond.[4] CLL, in particular, has been especially challenging; this disease is characterized by the uncontrolled expansion of B cells which infiltrate bone marrow, peripheral blood, and lymph nodes.[116] It has a very poor prognosis; allogeneic hematopoietic stem cell transplant is the only potentially curative treatment, and it carries with it significant risk of morbidity and mortality.[117] Only one CAR T cel (liso-cel) has regulatory approval for the treatment of CLL, and clinical outcomes are inconsistent.[118,119] Importantly, CLL is able to establish hypoxic niches characterized by overexpression of HIF-1α, suppressing the immune response through the upregulation of inhibitory cytokines and promoting the establishment of the T_{REG} compartment.[116,120] A 2018 study highlighted that anti-CD19 CAR T cells demonstrated durable antitumor responses in >90% of patients with r/r ALL, compared to 26% of patients with CLL.[121]

The limited response rate to anti-CD19 CAR therapy for CLL is often attributed to impaired fitness in the autologous cell product, including progressive exhaustion and diminished cytotoxicity.[122] Because of the known connection between tumor hypoxia and T cell dysfunction, and the potentially deleterious impact of supraphysiological oxygen levels on T cells *in vitro*, combined with the fact that CAR T cells as of today cannot consistently induce complete or durable

remission in the majority of patients with CLL, we decided to explore the possibility of culturing and interrogating CAR T cells at more physiological oxygen levels.

1.7 Summary

All told, incredible progress has been made in the field of immunotherapy over the last decades. Today, CAR T cells can cure patients who would otherwise die of their diseases. Even those patients who do not achieve durable responses to CAR cells may have years added to their lives thanks to the development of these remarkable therapies. However, there is a sore lack of CAR T cell therapies for solid tumors, and results in hematological malignancies are inconsistent, with the immunosuppressive microenvironment found in some of these diseases contributing to a lack of response. More work is needed to understand features of the TME that contribute to CAR T cell failure, and to develop CAR therapies that are not only able to penetrate inhospitable environments but survive and function within them.

2 Research aims

This work aimed to investigate different approaches to improve CAR T cell therapy for use in inhospitable environments, including solid tumors and hypoxic niches established by hematological malignancies. In pursuit of this, we evaluated the extent of trogocytosis and fratricide killing in an *in vitro* model of ovarian cancer using two MSLN-directed CAR constructs (**Paper I**), assessed the potential of a using cytostatic conditioning regimen with Treo and Flu together with MSLN-directed CAR T cells (**Paper II**), and examined the impact of oxygen restriction on CD19-directed CAR T cell phenotype and function (**Paper III**).

3 Materials and methods

This section contains an overview of the materials and methods used to generate CAR T cells and to interrogate their phenotypic and functional characteristics within the framework of each study. Detailed information can be found in the materials and methods sections of each respective paper.

3.1 Human materials

3.1.1 Healthy donor cells

Healthy donor buffy coats used as starting material for CAR T cell production in **Papers I–III** were purchased from the Karolinska University Hospital blood bank (Huddinge, Sweden).

3.1.2 Patient materials

Peripheral blood from individuals with CLL was made available to us through a collaboration with Karolinska University Hospital in Huddinge. Lymphocytes were isolated from these samples and used as starting material for CAR T cells in **Paper III**.

3.2 Ethical considerations

In **Paper III**, we performed research using blood from patients with CLL. The collection and use of these materials was approved by the Swedish Ethical Review Agency (Etikprövningsmyndigheten DNR 00-138). Patients gave their informed consent in accordance with the Declaration of Helsinki before inclusion. Samples were collected by the Anders Österborg group (Department of Oncology-Pathology, Karolinska institutet).

3.3 Modeling hypoxia

In the context of **Papers II and III**, "normoxia" refers to 21% O_2 and "hypoxia" to 2% O_2. We utilized a ICO50 CO_2 incubator (Memmert) which replaces oxygen with nitrogen to control the oxygen tension within the chamber.

3.4 CAR production

3.4.1 Production of CAR vector

In **Papers I and II**, we utilized two second-generation anti-MSLN CAR constructs: one bearing a CD28 co-stimulatory domain (M28ζ), and the other bearing a 4-1BB

co-stimulatory domain (MBBζ). Additionally, in **Paper II**, we utilized an anti-MSLN CD28 co-stimulated 1xx CAR (M1xx). The anti-MSLN CAR constructs are shown in Figure 7. The retroviral vectors bearing the genetic material of these CARs were produced using the packaging cell line RD114, generously provided by Professor Michel Sadelain (Memorial Sloan Kettering Cancer Center, New York City, NY, USA). In **Paper III**, the packaging cell line PG13 was used to produce the retroviral vector bearing the genetic material of the second-generation anti-CD19 CD28-co-stimulated CAR. This packaging cell line was kindly provided by Professor Steven Rosenberg (National Cancer Institutes, Bethesda, MD, USA).

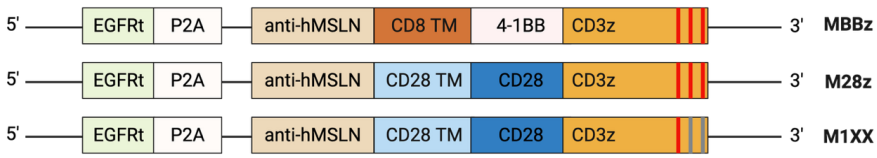

Figure 7. Anti-MSLN CAR constructs

3.4.2 CAR T cell transduction

PBMCs were isolated from healthy donor or patient buffy coats using Ficoll Paque (Cytiva) density gradient centrifugation medium according to manufacturer instructions. PBMCs were cultured in AIM V medium (Invitrogen, ThermoFisher Scientific) supplemented with 5% human AB serum (HS, Sigma-Aldrich) (**Papers I – III**) or 10% fetal bovine serum (FBS, Hyclone) (**Paper III**). Cells were seeded at a density of 0.5-1E6/mL and activated using 50 ng/mL anti-CD3 monoclonal antibody (OKT3, Biolegend) and 300 IU/mL IL-2 (Proleukin, Novartis) and incubated at 37°C, 5% CO_2, and 21% O_2 (**Papers I-III**) or 2% O_2 (**Papers II and III**). Two days after activation, retroviral supernatant was added to 24-well non-tissue culture-treated plates that had been pre-coated with RetroNectin (TaKaRa) and blocked with a solution of 2.5% human serum albumin. Plates were centrifuged for 2 hours at 2000 G and 32°C, binding the viral particles to the well. Cells were then added to the wells at a density of 2E6 per well and centrifuged for 30 minutes at 2000 G and 32°C. The following day, cells were removed from transduction plates and seeded in flasks in fresh expansion medium. Cells were counted and medium refreshed every second day for up to six days, at which point cells were frozen for later use. CAR expression was confirmed by flow cytometry three or four days after transduction.

3.5 Human cancer cell lines

Multiple human ovarian cancer cell lines transduced to express MSLN were used as CAR T cell targets: OVCAR-3 (epithelial cells from malignant ascites in ovarian adenocarcinoma, ATCC, HTB-161; **Paper I**), SKOV-3 (epithelial cells from ovarian adenocarcinoma, ATCC, HTB-77; **Papers I and II**), and OVCAR-4 (high-grade serous ovarian adenocarcinoma, gifted by Dr. Kaisa Lehti, Department of Microbiology, Tumor and Cell Biology, Karolinska institutet; **Paper II**). K562 cell line (chronic myelogenous leukemia lymphoblasts; kindly provided by Dr. Steven Feldman, National Cancer Institutes, Bethesda, MD, USA) transduced to express NGFR was used in **Papers I and III** as a non-MSLN-expressing control, and transduced to express CD19 in **Paper III** as a CAR T cell target.

3.6 Flow cytometry

Most of the results presented in this thesis were generated using flow cytometry, which allows us to detect extra- and intracellular receptors, transcription factors, and more at the single-cell level through the use of fluorochrome-conjugated antibodies or dyes which bind to targets of interest.

Three systems make up flow cytometers: fluidics, optics, and electronics. Cells are taken up and transported via the fluidics system through the optics system, which is made up of lasers (excitation optics) and photomultiplier tubes and photodiodes (collection optics).[123] Excitation and collection optics generate light signals which are passed through a series of dichroic filters, which block certain light signals while allowing others to pass through; they can also function as mirrors to reflect the light at a 90° angle, redirecting the light to pass through another series of filters.[124] There are three filter types: short pass, which lets through signals shorter than a given wavelength; long pass, which lets through signals longer than a given wavelength: and band pass, which lets through signals within a specific wavelength range. The electronics system converts signals into digital signatures, which can then be interpreted by a computer.[123]

3.6.1 Fluorescence-activated cell sorting

To ensure antigen expression (MSLN, **Papers I and II**, and CD19, **Paper III**) on tumor cell line, we performed fluorescence-activated cell sorting (FACS). This flow cytometry-based modality works by labeling cells with a fluorescence-conjugated antibody and then passing them through a flow cytometer, and using a gate to select cells positive above a certain threshold for the antigen. The sorter

is then able to separate cells positive for the antigen from cells negative for the antigen and deflect them into collection vessels.[123]

3.6.2 CAR transduction efficiency

Transduction levels of MSLN and CD19 CAR were assessed after transduction and expression levels were confirmed prior to initiating experiments. CAR expression was indirectly measured using biotin-conjugated antibodies. The MSLN CAR constructs used in **Papers I and II** incorporate a truncated EGFR (EGFRt) domain, which we detected by staining with a biotinylated anti-EGFR-antibody (R&D Systems) for 20 minutes followed by a staining with PE-conjugated Streptavidin (Biolegend), which binds to biotin. In **Paper III**, CD19 CAR expression was detected by staining with a biotin-conjugated anti-CD19 CAR antibody fragment (Fab) (Jackson Immunoresearch) for 40 minutes and subsequently staining with PE-conjugated Streptavidin. .

3.6.3 Intracellular cytokine staining

Intracellular cytokine staining (ICS) is a classical technique for evaluating T cell functionality. In **Papers I–III**, CAR T cells were cultured with their respective targets for six hours in the presence of the protein transport inhibitors brefeldin A (Sigma-Aldrich) and GolgiStop (containing Monensin; BD Biosciences). The protein transport inhibitors block cytokine secretion, which allowed us to subsequently detect presence of accumulated inflammatory cytokines and the degranulation marker CD107a using fluorescence-conjugated antibodies. Untransduced cells co-cultured with antigen-expressing tumor cell line and CAR T cells co-cultured with antigen-negative tumor cell line were included as controls.

3.6.4 Mitochondrial characterization

In **Papers II and III**, we used fluorescent dyes to characterize mitochondrial fitness in CAR T cells by flow cytometry. Tetramethylrhodamine ethyl ester (TMRE) is used to measure mitochondrial membrane potential, MitoSOX to assess levels of mitochondrial ROS (mROS), and MitoTracker Green (MTG) to measure mitochondrial mass (all mitochondrial probes: ThermoFisher Scientific). Mitochondria are highly dynamic organelles, labeling with fluorescent dyes can only take a snapshot of a specific moment in time. Other methods, such as the SeaHorse Real-Time Cell Metabolic Analysis (Agilent), allow real-time testing of oxidative consumption rate and spare respiratory capacity, and thus offers a more comprehensive evaluation of mitochondrial fitness; however, this method was not

compatible with our experimental setup. Confocal microscopy also has exciting applications for mitochondrial assessment, allowing for assessment of real-time changes in mitochondrial morphology; however, this modality was not an option within the confines of our experimental setup. Mitochondrial dyes are time-tested, robust markers of different characteristics that together provide a framework for assessing mitochondrial fitness within a cell; we used them primarily to perform one-to-one comparisons of donor-matched cells exposed to different oxygen levels in order to characterize the impact of oxygen availability on mitochondrial fitness.

3.7 Cytotoxicity assays

Throughout the three papers included in this thesis, we use multiple principally distinct assays to evaluate CAR T cell cytotoxicity. The respective killing assays were selected depending on experimental setups.

3.7.1 LDH cytotoxicity assay

In **Paper I**, we used a lactate dehydrogenase (LDH)-based assay (CyQUANT LDH Cytotoxicity Assay, Thermo Fisher Scientific) to assess effector function of mixed unsorted $CD4^+/CD8^+$ CAR T cells, along with sorted $CD4^+$ and $CD8^+$ CAR T cells against target SKOV-3 and OVCAR-3 targets, which were either unsorted ($MSLN^{neg/low}$) or sorted to express high MSLN. LDH is released by damaged cells, catalyzing the conversion of lactate to pyruvate; in this reaction, oxidized nicotinamide adenine dinucleotide (NAD^+) is reduced to NADH, which in turn leads to the generation of a red formazan product that can be measured using a plate reader (in our case, the ClarioSTAR plate reader, BMG Labtech).

3.7.2 Flow cytometry-based killing

In **Paper I**, we performed fratricide killing assays using flow cytometry. We transduced "target" T cells to express MSLN, and sorted them by FACS to achieve different levels of MSLN expression: $MSLN^{high}$, $MSLN^{low}$, and $MSLN^{high/low}$ (a 1:1 mix of $MSLN^{high}$ and $MSLN^{low}$). We labeled all target T cells with CellTrace Violet (Invitrogen), a proliferation marker, in order to be able to subsequently identify parental $MSLN^+$ T cells. We then co-cultured target T cells with CAR T cells at a 2:1 CAR:target ratio for four and 24 hours, and at the end of co-culture acquired cells by flow cytometry, calculating cytotoxicity by measuring total cells excluding CTV-labeled, 7AAD-negative (live) CAR-expressing cells. Untransduced T cells

were included as a control and killing was normalized to the number of CTV-expressing, 7AAD-negative untransduced cells present after co-culture.

3.7.3 One-Glo™EX Luciferase Assay System

To assess CAR T cell killing capacity in **Papers II and III**, we used the One-Glo™EX Luciferase Assay System. In this experiment, target cells were K562 transduced to express CD19 and firefly luciferase; when these cells die, they stop expressing luciferase and become less fluorescent. We co-cultured target cells with CAR T cells expressing a relevant CAR at different effector:target ratios for a period of 24 hours. CAR T cell killing was normalized to killing by donor-matched untransduced T cells.Readout was performed on a ClarioSTAR plate reader.

3.7.4 Chromium^{51} (Cr^{51})-based cytotoxicity

In **Papers I and III**, we used Cr^{51}-based cytotoxicity to evaluate CAR T cell cytotoxic function. This assay was performed by labeling K562 cells expressing target antigen with Cr^{51}; following co-culture with CAR T cells, chromium release was detected on a 1450 MicroBeta liquid scintillation counter (Perkin Elmer) as counts per minute. Killing was normalized to the negative control, untransduced T cells co-cultured with antigen-expressing K562.

3.8 Data analysis and statistics

In all included papers, flow cytometry was analyzed using FlowJo software (FlowJo). Figure production and statistical analysis were performed using GraphPad Prism v10 (Prism). Wilcoxon matched pairs signed rank tests were used to compare two groups of paired samples, and Friedman tests followed by Dunn's multiple comparisons tests were used to compare three or more. Mann-Whitney tests were used to compare two groups of unpaired samples, and Kruskal-Wallis tests were used to compare three or more.

4 Results

Here, a concise overview of selected results from each included paper is provided. Please see the respective papers for full results.

4.1 Paper I: Trogocytosis and fratricide killing impede MSLN-directed CAR T cell functionality

In this *in vitro* study we evaluated CD28- (M28ζ) and 4-1BB-co-stimulated anti-MSLN CAR T cells (MBBζ) for functional and phenotypic responses to challenge with MSLN-expressing ovarian cancer cell lines, with specific focus placed on trogocytosis impacts CAR T cell phenotype and function.

4.1.1 Phenotypic characterization and cytokine production

We found that M28ζ CAR T cells contained significantly fewer $CD4^+$ but significantly more $CD8^+$ cells compared to donor-matched MBBζ CAR T cells. MBBζ CAR T cells contained significantly more $CD4^+$ cells than $CD8^+$. We found no significant differences in frequency of memory phenotype subsets between CAR constructs, but did note significantly more T_{EMRA} cells among M28z and significantly more T_{CM} among MBBζ compared to donor-matched untransduced cells. In Figure 8, we demonstrate that cytokine release in $CD4^+$ cells of M28ζ and MBBζ was largely comparable, with MBBζ $CD4^+$ displaying a slight but significant increase in degranulation (measured by CD107a) compared to M28ζ. In the $CD8^+$ compartment, differences were greater: M28ζ produced significantly more IFNγ, TNF, and IL-2 relative to MBBζ.

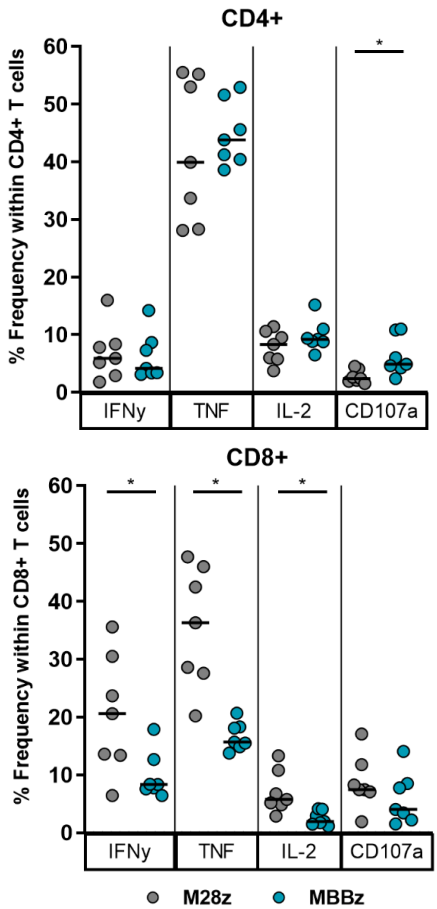

Figure 8. Cytokine production by CD4 (top) and CD8 (bottom) anti-MSLN CAR T cells.

4.1.2 Trogocytosis

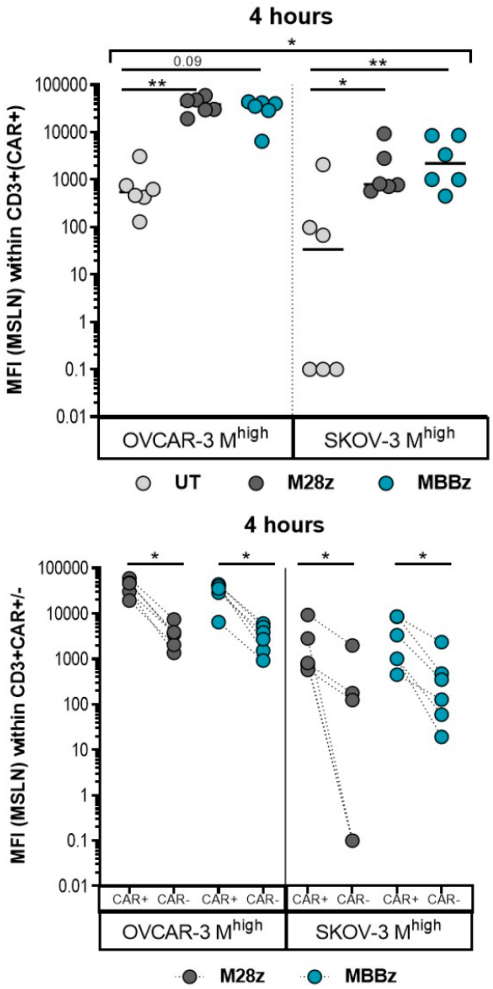

Figure 9. Trogocytosis in UT, M28z, and MBBz cells after 4 hours of co-culture (top); difference in level of trogocytosis in CAR+ and CAR- fractions of cell product (bottom).

We assessed the occurrence of trogocytosis between M28ζ and MBBζ CAR T cells co-cultured with MSLN^{high} OVCAR-3 and SKOV-3. Trogocytosis was detected as early as four hours after start of co-culture; interestingly, CAR T cells co-cultured with OVCAR-3 expressed significantly more MSLN than those co-cultured with SKOV-3. Further, we found that MSLN expression was significantly higher in CAR$^+$ than corresponding CAR$^-$ cells, suggesting that trogocytosis is mediated by CARs; shown in Figure 9. We also found that CD4$^+$ CAR T cells expressed significantly more MSLN after co-culture than corresponding CD8$^+$ CAR T cells. Notably, viability of trogocytotic CAR T cells was inversely correlated with MSLN expression levels, indicative of fratricide killing. Throughout, we found no significant difference in the level of trogocytosis mediated by M28ζ CAR T cells compared to MBBζ CAR T cells.

4.1.3 Expression of coinhibitory molecules

We evaluated expression of the inhibitory receptors (coinhibitory molecules, CIM) PD-1, LAG-3, and TIM-3 relative to MSLN acquisition. We found that MSLN$^+$ CAR T cells were significantly more triple positive (TP; PD-1$^+$/LAG-3$^+$/TIM-3$^+$) compared to MSLN$^-$ CAR T cells. The reverse was also true; MSLN$^-$ CAR T cells were significantly more triple negative (TN) compared to MSLN$^+$ CAR T cells. These results are summarized in Figure 10.

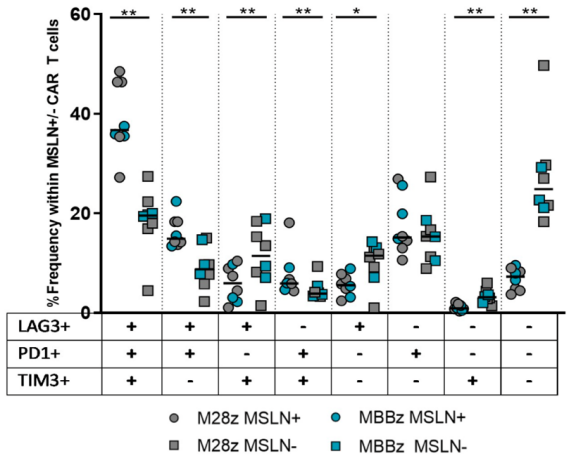

Figure 10. CIM expression in MSLN+ and MSLN- CAR T cells

We also interrogated expression of inhibitory receptors on M28ζ and MBBζ CAR T cells in response to co-culture with MSLN^{high} SKOV-3 and OVCAR-3 and found that co-expression was highly dynamic. At the start of co-culture, the frequency of TP cells was significantly higher in M28ζ compared to MBBζ, and the frequency of TN cells was significantly lower. After 24 hours of antigen exposure, frequency of TP cells increased among both M28ζ and MBBζ; more so in response to co-culture with OVCAR-3 cells. Frequency of TP cells fell, and TN cells rose in absence of antigen stimulation. On restimulation, frequency of TP cells rebounded among both M28ζ and MBBζ. Significantly, we found that co-culture with OVCAR-3 led to a greater increase in frequency of TP cells compared to co-culture with SKOV-3. These results are shown in Figure 11.

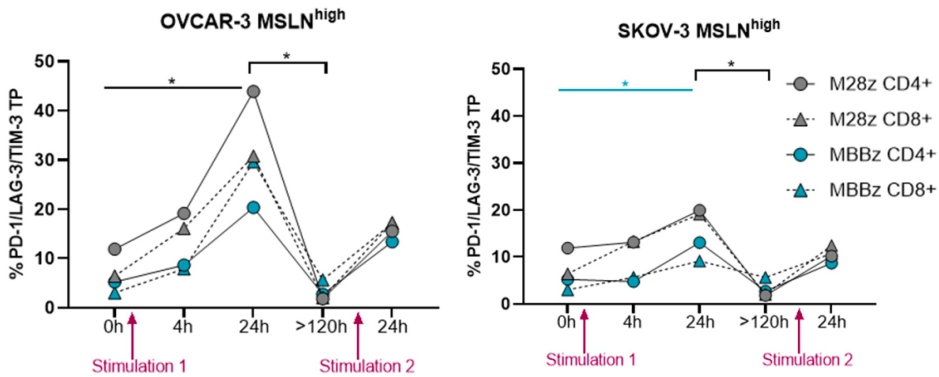

Figure 11. Frequency of TP cells in M28z and MBBz co-cultured with OVCAR-3 (left) and SKOV-3 (right)

4.1.4 Association between trogocytosis, cytotoxic capacity, and expression of coinhibitory molecules

We went on to perform unbiased hierarchical clustering using data sets generated in the same experiment – cytotoxicity, MSLN expression (trogocytosis), and CIM expression. Analysis revealed four distinct clusters – A1-4, which clustered primarily according to CIM expression and/or target cell. Notably, clusters did not separate according to CAR construct. Characteristics of these clusters are summarized in Table 4.

Cluster	No. of donors in cluster	Characteristics	Cytotoxicity	Trogocytosis	CIM
A1	2	Only donors co-cultured with $MSLN^{high}$ OVCAR-3	No OVCAR-3 lysis	↑↑↑ (median 95% $MSLN^{+}$)	↑LAG3+ vs A3, A4
A2	5	Only donors co-cultured with $MSLN^{high}$ OVCAR-3			↑LAG3+ vs A3, A4
A3	7	Mainly (6/7) donors co-cultured with $MSLN^{high}$ SKOV-3	Highest level of target cell lysis		↓LAG-3+/TIM-3+ DP vs A4 ↑PD-1+/TIM-3+ vs A4
A4	10	Donors co-cultured with either $MSLN^{high}$ OVCAR-3 or SKOV-3			↓PD1+ vs A2, A3

Table 4. Summary of unbiased hierarchical clustering of cytotoxicity, trogocytosis, and CIM expression.

4.2 Paper II: Impact of fludarabine and treosulfan on ovarian tumor cells and mesothelin chimeric antigen receptor T cells

In this paper, we performed *in vitro* experiments in pursuit of evaluating the potential of Flu and Treo as potential combination therapy together with three MSLN-directed CAR T cell constructs (M28ζ, MBBζ, and M1xx) directed against MSLN-expressing ovarian cancer cell line.

4.2.1 Impact of Flu and Treo on CAR T cell mitochondrial fitness

We performed an initial assessment of CAR T cell viability following co-culture with Flu or Treo, and found that after 24 hours viability had decreased to median 50%, and median 12% after 48 hours. We assessed the impact of Treo or Flu on mitochondrial fitness of surviving cells in each of the three CAR constructs using the mitochondrial probes MitoSOX, TMRE, and MTG, shown in Figure 12. Treo had no apparent impact on mitochondrial fitness; Flu, on the other hand, led to a slight but significant increase in MitoSOX in $CD8^+$ M28z CAR T cells compared to untreated donor-matched cells; Flu also led to an increase in MTG expression in both $CD4^+$ and $CD8^+$ M1xx CAR T cells compared to untreated cells.

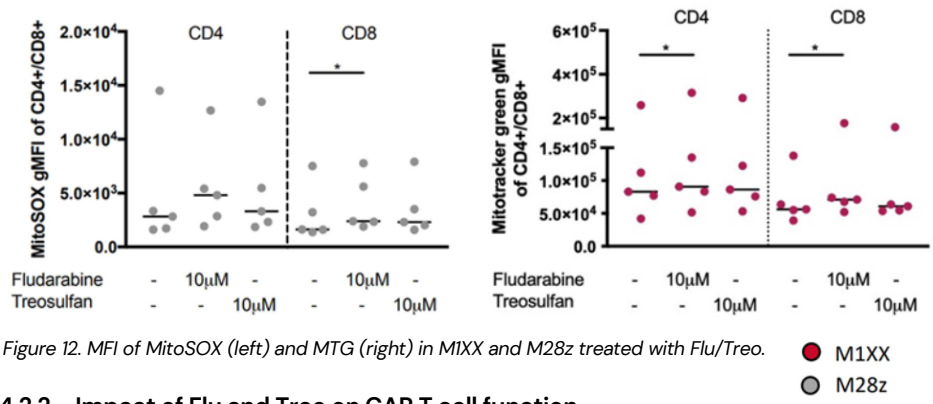

Figure 12. MFI of MitoSOX (left) and MTG (right) in M1XX and M28z treated with Flu/Treo.

4.2.2 Impact of Flu and Treo on CAR T cell function

We investigated the impact of Flu, Treo, and Flu + Treo on MSLN CAR T cell cytokine secretion and degranulation and found no significant differences in level of IFNγ, IL-2, TNF, or CD107a produced by M1xx, M28ζ, or MBBζ CAR T cells after a six-hour co-culture in normoxia, or M28ζ CAR T cells in hypoxia. As shown in Figure 13, in normoxia we found a significant inhibition of M28ζ CAR T cell cytotoxicity

treated with Flu + Treo compared to untreated CAR T cells. This difference was not seen in hypoxia.

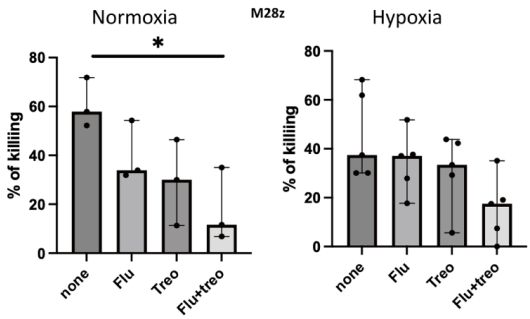

Figure 13. Cytotoxicity of CAR T cells co-cultured with Flu/Treo.

4.3 Paper III: Production of functional CD19 CAR T cells under hypoxic manufacturing conditions

In Paper III, we assessed the potential of producing CD19-directed CAR T cells in a low-oxygen environment, hypothesizing that this would select for a population of hypoxia-resistant CAR T cells, or generate a more hypoxia-tolerant cell product. CAR T cells produced in 21% O_2 and 2% O_2 are referred to as ^{Nor}CAR and ^{Hyp}CAR, respectively.

4.3.1 Memory phenotype following production and stimulation

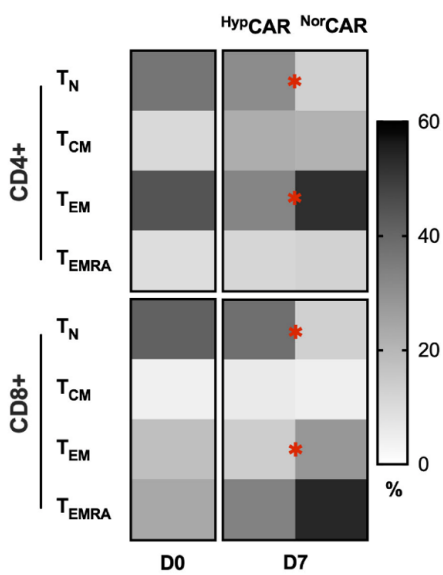

Figure 14. Memory phenotype of ^{Nor}CAR and ^{Hyp}CAR T cells 7 days following activation.

We found that restricted oxygen availability quickly impacted CAR T cell product memory differentiation, as shown in Figure 14. After CAR production, CD4$^+$ and CD8$^+$ ^{Hyp}CAR product contained significantly more T_N cells, and significantly fewer T_{EM} cells, than ^{Nor}CAR product.

We assessed memory phenotype subsequent to one (T1) and two (T2) stimulations with cognate antigen. Here, co-cultures were performed in 21% or 2% O_2; CAR product produced in 21% or 2% O_2 and stimulated in an oxygen tension congruent to production conditions are

referred to as Nor-Nor and Hyp-Hyp, respectively; CAR product produced in 21% O_2 and interrogated in 2% O_2 is referred to as Nor-Hyp. We found several differences in relative size of the different memory subsets between oxygen conditions, summarized in Figure 15. Most notably, in $CD4^+$ and $CD8^+$ $^{Hyp-Hyp}$CAR product at T1, there were significantly increased levels of T_N relative to $^{Nor-Hyp}$CAR and $^{Nor-Nor}$CAR products. This difference persisted between $CD4^+$ and $CD8^+$ $^{Hyp-Hyp}$CAR and $^{Nor-Nor}$CAR product at T2. Further, in all conditions, $^{Hyp-Hyp}$CAR product contained significantly fewer T_{EM} cells than $^{Nor-Nor}$CAR product.

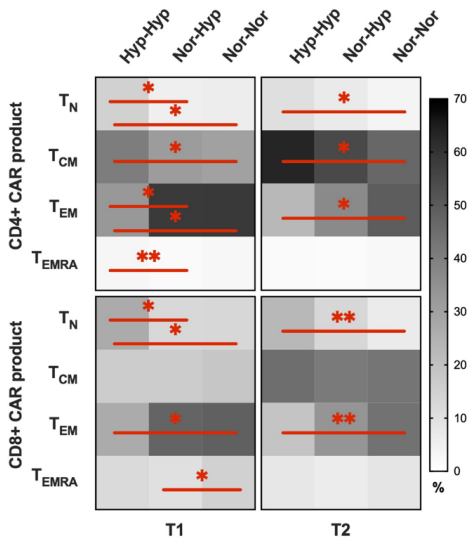

Figure 15. Memory phenotype of CAR product after stimulation.

4.3.2 Functional consequences of production in hypoxia

We performed ICS using Nor-Nor, Nor-Hyp, and Hyp-Hyp setups. $CD4^+$ $^{Hyp-Hyp}$CAR product secreted slightly but significantly higher levels of IL-2 and TNF relative to corresponding Nor-Nor cells, shown in Figure 16. We noted no significant differences in cytokine production between oxygen conditions in the $CD8^+$ compartment.

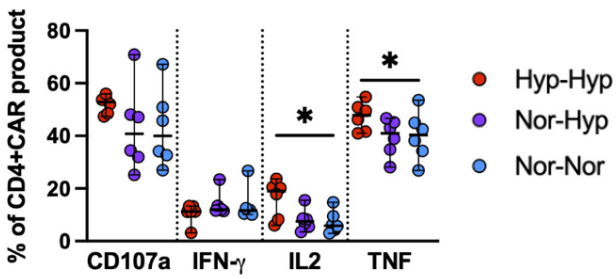

Figure 16. Cytokine production in CD4+ CAR product.

We used the same experimental setup to evaluate cytotoxicity, as shown in Figure 17. We found that at a 1:2 CAR:target ratio, $^{Nor\text{-}Nor}$CAR product demonstrated significantly increased target cell lysis relative to $^{Hyp\text{-}Hyp}$CAR product. At the more stringent 1:4 and 1:10 CAR:target ratios, there was no difference in killing capacity between oxygen conditions.

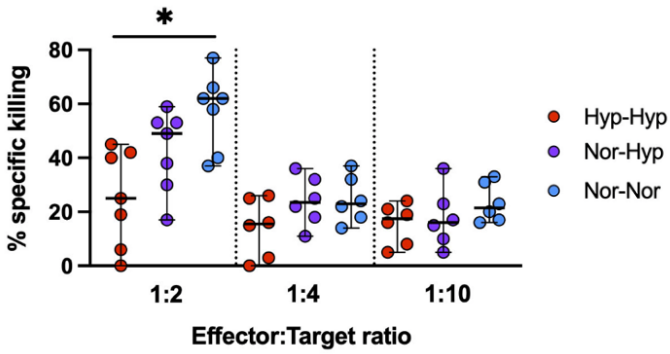

Figure 17. Cytotoxicity between oxygen conditions at various E:T ratios.

4.3.3 Impact of O_2 tension on CAR product mitochondrial fitness

As shown in Figure 18, in CAR product derived from healthy donor cells, we found that CD8$^+$ $^{Nor\text{-}Nor}$CAR product expressed significantly higher levels of TMRE compared to the corresponding $^{Hyp\text{-}Hyp}$CAR product at both T1 and T2. We found no difference between oxygen conditions in the CD4$^+$ compartment.

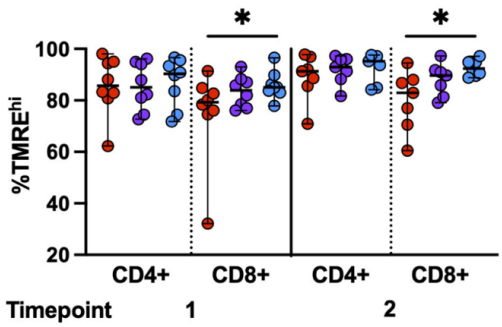

Figure 18. Frequency of polarized mitochondria in CD8$^+$ CAR product after stimulation.

In CAR product derived from patients with CLL, mitochondrial fitness was assessed at a single timepoint without stimulation. In Figure 19, we show that MTG was significantly increased in CD4$^+$ ^{Hyp}CAR cells compared to the corresponding ^{Nor}CAR cells. TMRE was significantly increased in ^{Hyp}CAR product in both CD4$^+$ and CD8$^+$ compartments relative to ^{Nor}CAR product. Finally, we found

increased levels of MitoSOX in both CD4+ and CD8+ HypCAR product compared to NorCAR product.

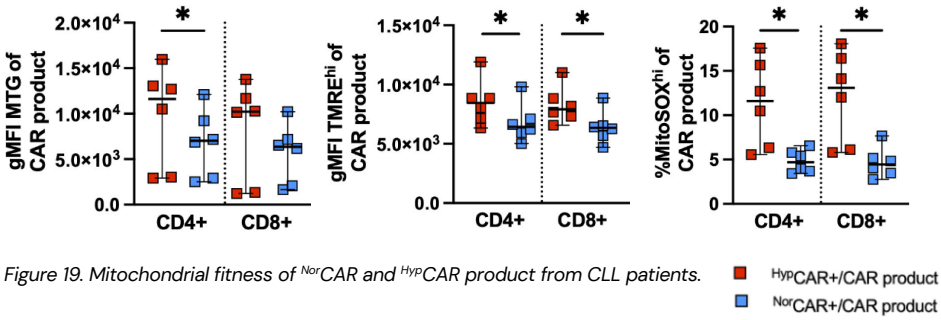

Figure 19. Mitochondrial fitness of NorCAR and HypCAR product from CLL patients.

5 Discussion

The overarching aim of this thesis was to investigate approaches to enhancing CAR T cells for use in inhospitable environments, with a focus on understanding phenotypic and functional differences between M28ζ and MBBζ CAR T cells and how they contribute to trogocytosis (**Paper I**), the synergistic potential of combining Flu and Treo with anti-MSLN CAR T cells (**Paper II**), and the impact of producing anti-CD19 CAR T cells in an oxygen-deficient environment (**Paper III**) in order to prime them to meet an inhospitable TME.

5.1 The impact of trogocytosis and fratricide killing on anti-MSLN CAR T cell phenotype and functionality

In **Paper I**, we identified early occurrence of trogocytosis in MSLN-expressing ovarian cancer cell line co-cultured with M28ζ and MBBζ CAR T cells, but found no significant difference in the level of antigen loss mediated by either construct. Interestingly, it has recently been demonstrated the protein's transmembrane domain is a key determinant of whether trogocytosis will occur; long and "stretchable" transmembrane domains, including transmembrane domains derived from CD8 and CD28, were effectively trogocytosed compared to "non-stretchable" transmembrane domains (for instance derived from CD45).[97] Here, we primarily evaluated the horizontal transfer of MSLN from tumor cells to CAR T cells, and not anti-MSLN CAR from CAR T cells to tumor cells, but the level of trogocytosis of the CAR molecule to other cells can be surmised to be comparable (and likely high) given that the M28ζ and MBBζ CAR used here have a "stretchable" CD28 and CD8 transmembrane domain, respectively.

While we did not find any significant differences in the degree of trogocytosis mediated by M28ζ relative to MBBζ CAR T cells, *in vivo* work from our group using an orthotopic model of ovarian cancer demonstrated significantly higher trogocytosis mediated by MBBζ cells compared to M28ζ CAR T cells 10-25 days after CAR infusion.[125]

We found that M28ζ displayed significantly enhanced spheroid infiltration and cytotoxicity in $MSLN^{high}$ SKOV3 spheroids compared to MBBζ cells, and only M28ζ CAR T cells were able to infiltrate and effect cytotoxicity in $MSLN^{low}$ SKOV3 spheroids. This is in line with previous observations that CD28-co-stimulated CAR T cells are more potently cytotoxic than 4-1BB-co-stimulated CAR T cells, even in low-antigen settings.[100]

Trogocytosis has been associated with higher co-expression of inhibitory receptors,[100] as we found here; co-expression of PD-1, LAG-3, and TIM-3 is a well-known indicator of T cell exhaustion, suggesting that CAR T cells affected by trogocytosis are more prone to phenotypic exhaustion.

The observation that co-culture with OVCAR-3 led to a greater increase in the level of TP cells compared to SKOV-3 cells in the same culture system and within the same time frame suggests that tumor cell-intrinsic characteristics impact the level of trogocytosis that occurs; this has been observed previously but is not fully understood. Evidence suggests that tumor-derived factors may promote trogocytosis through the activation of the transcription factor activating transcription factor-3, which is a stress-response gene activated in the TME in response to, for instance, restricted access to oxygen.[98]

5.2 The synergistic potential of combining Flu and Treo with anti-MSLN CAR T cells

In **Paper II**, we found that exposure to Treo, Flu, or Treo/Flu had no significant impact on MSLN-directed CAR T cells' functional capacity or mitochondrial fitness. Together, Treo + Flu are commonly used prior to allogeneic hematopoietic stem cell transfusion;[104] to our knowledge, our paper is the first to evaluate this combination as a potential lymphodepleting regimen prior to anti-MSLN CAR T cells for ovarian cancer.

Pretreatment with chemotherapy before CAR T cell infusion has been demonstrated to significantly improve outcomes in hematological malignancies through enhanced CAR T cell expansion and engraftment, as well as TME polarization. Fludarabine is already widely used as lymphodepletion prior to CAR therapy for hematological malignancies, usually in combination with Cy.[126] Treo is not commonly used prior to CAR T cell therapy; it has been demonstrated to have clinical activity against a number of solid tumors,[127] including ovarian cancer, where it primarily used in patients with end-stage disease.[128]

Chemotherapeutic preconditioning for solid tumor-directed CAR T cells is less well-characterized than for hematological malignancies, but available data indicates that it is likely of pivotal importance for successful CAR T cell therapy. In an immunocompetent murine model of PSCA-expressing prostate cancer, Murad and colleagues demonstrated that treatment with anti-PSCA CAR T cells alone was unable to eradicate tumors, but preconditioning with Cy prior to

CAR infusion led to significantly improved infiltration of both CAR T cells and endogenous T cells into tumors, along with polarization of an M2 macrophage population to a pro-inflammatory M1 phenotype, and increased antigen expression on tumor cells, resulting in enhanced tumor control. Investigators highlighted the fact that the addition of Cy, apart from simply "creating space" for the infused CAR T cells to survive and thrive within the lymphocyte compartment, directly induced changes in the TME which promoted CAR T cell function.[129] Sagie and colleagues connected lymphodepletion with Cy and Flu prior to ACT to increased intratumoral antigen processing and presentation,[130] making tumors more visible to adoptively transferred cells.

5.3 The impact of producing anti-CD19 CAR T cells in an oxygen-deficient environment

In **Paper III**, we demonstrate accumulation of naïve-like cells in hypoxia after production and the persistence of a naïve-like population in $CD4^+$ CAR product in hypoxia. As discussed, infusing naïve-like CAR T cells is associated with favorable clinical outcomes, but here, we cannot exclude that the accumulation of naïve-like cells is actually due to an impairment that prevents further differentiation. We cannot draw any conclusions as to what this might translate to *in vivo*.

In terms of mitochondrial fitness, we demonstrated overall high levels of $TMRE^{hi}$ frequency in $CD4^+$ and $CD8^+$ CAR product after one and two stimulations with cognate antigen, but find significantly increased levels in $CD8^+$ $^{Nor-Nor}$CAR product compared to $^{Hyp-Hyp}$CAR product at both timepoints. This is potentially important – reduced mitochondrial membrane potential has been shown to be associated with enhanced cytotoxicity and persistence in adoptively transferred $CD8^+$ T cells.[28,131] Interestingly, we found no significant difference in mitochondrial membrane potential between $CD8^+$ $^{Nor-Nor}$CAR product and $^{Nor-Hyp}$CAR product, which might suggest that durable oxygen-related changes to cellular metabolism occur during CAR production.

We found a significant increase in CD98 expression in $CD8^+$, but not $CD4^+$, $^{Hyp-Hyp}$CAR product compared to $^{Nor-Hyp}$CAR and $^{Nor-Nor}$CAR product after two stimulations with cognate antigen. CD98, a large amino acid transporter, is important for T cell differentiation and has been shown to be associated with highly glycolytic cells.[29]

In a 2019 report, van Bruggen and colleagues delineated a mitochondrial profile associated with resting CLL-derived $CD8^+$ T cells: increased

mitochondrial respiration, membrane potential, and ROS levels. This profile was associated with impaired mitochondrial biogenesis. In CAR product derived from patients with CLL, we found significantly increased mitochondrial membrane potential and mROS in both $CD4^+$ and $CD8^+$ hypoxic compartments compared to normoxic cells. In the absence of a healthy donor control, we can speculate that culture in hypoxia led to further disturbance of the mitochondrial metabolism of these cells. We did also observe significantly increased mitochondrial mass in $CD4^{+\ Hyp}$CAR product compared to $CD4^{+\ Nor}$CAR product, a trait which van Bruggen and colleagues found was associated with complete response to CD19 CAR T cell therapy, albeit in the $CD8^+$ compartment.[122]

6 Conclusions

From the work presented here and in the accompanying articles, we conclude:

- Trogocytosis is mediated at similar levels by CD28- and 4-1BB-co-stimulated anti-MSLN CAR T cells. Antigen loss through trogocytosis, along with fratricide killing subsequent to antigen acquisition by CAR T cells, is likely an important contributing factor to the lack of success seen in treating solid tumors with MSLN-directed CAR T cells.
- There is a rationale for using Treo and Flu combination therapy as pretreatment for anti-MSLN CAR T cells in ovarian cancer, based on the fact that they mediate tumor cell death without contributing to antigen loss, and that they do not have an outsize negative impact on anti-MSLN CAR T cell fitness.
- Production in hypoxia confers long-lasting phenotypic qualities on CD19-directed CAR T cells that may be favorable in a clinical setting, including the enrichment of a naïve-like cohort in ^{Hyp}CAR product.

7 Points of perspective

CAR T cell therapy has been established as a powerful modality in the cancer treatment arsenal, but there are several hurdles that pose very real challenges to successful CAR therapy, especially in the context of inhospitable disease environments.

Trogocytosis is demonstrated to contribute to antigen loss and loss of CAR expression in both hematological malignancies and solid tumor diseases. In **Paper I**, we highlight the challenge posed by antigen loss through trogocytosis. We find that CD28-co-stimulated CAR T cells demonstrate enhanced cytotoxicity relative to 4-1BB co-stimulated CAR T cells despite the fact that they mediate the same degree of trogocytosis (target to effector), which raises interesting questions, for instance whether a CAR with powerful effector function is able to overcome some of the negative effects of trogocytosis. Further, trogocytosis occurred to a greater extent in M28ζ and MBBζ CAR T cells co-cultured with OVCAR-3 than SKOV-3, despite similar levels of initial MSLN expression. The underlying mechanisms regulating trogocytosis are not yet fully understood, and more work is needed to map out factors that promote and inhibit trogocytosis, respectively. Understanding trogocytosis is an important step on the road to developing robust CAR therapies.

The results from **Paper II**, combined with the demonstrated utility of Treo and Flu in the context of ovarian cancer and CAR T cell pretreatment, respectively, warrant further investigation into Treo + Flu as a potential pretreatment for MSLN-directed CAR T cells for ovarian cancer. Identifying which drug(s), dosages, and timing of treatment (i.e. as bridging, lymphodepletion, or neoadjuvant/adjuvant) enhance CAR T cell function for solid tumors is essential, not least because of the fact that these therapies are readily available, they are inexpensive, and they have often been validated for use in several diseases.

In **Paper III**, we provide support for culture in hypoxia or low-physiological oxygen conditions as a potential method for enhancing CAR product for use in oxygen-restricted environments. We and others have demonstrated that the relatively simple action of restricting oxygen tension during production is enough to confer long-lasting phenotypic and functional changes on CAR T cells that may well give them an advantage *in vivo*, though results are not entirely unambiguous, and it is unclear whether it is due to the selection of a hypoxia-tolerant subset or "priming" that prepares them to meet an oxygen-restricted

intratumoral environment. Further work is needed to determine clinical translation of these findings and to calibrate optimal oxygen levels and exposure times.

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