

From the department of Oncology-Pathology  
Karolinska Institutet, Stockholm, Sweden

# **DEFINING THE POTENTIAL OF MESOTHELIN-DIRECTED CAR T CELLS FOR THE TREATMENT OF OVARIAN CANCER**

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# Defining the potential of mesothelin-directed CAR T cells for the treatment of ovarian cancer

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

**Esther Schoutrop**

The thesis will be defended in public at H2 Grön, Alfred Nobels Allé 23, Karolinska Institute, Campus Flemingsberg (Huddinge) on Friday October 21<sup>st</sup>, 2022 at 14:00

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## POPULAR SCIENCE SUMMARY OF THE THESIS

Approximately 1 out of every 100 women will receive the devastating diagnosis of ovarian cancer during their lifetime. Ovarian cancer is sometimes referred to as the ‘silent killer’, since the early signs of disease are not easily recognized. Half of the patients diagnosed with ovarian cancer will die from the disease within 5 years, making ovarian cancer one of the most dangerous cancers for women worldwide<sup>1 2</sup>. The treatment regimen for ovarian cancer is invasive, usually consisting of extensive surgery combined with chemotherapy. Despite the harsh treatment regimens, the majority of patients will suffer from disease relapse<sup>3</sup>. New therapies are urgently needed for the large number of women being failed by the current treatment options for ovarian cancer.

Immunotherapy has emerged as a powerful treatment strategy in the fight against cancer and has even been rewarded with a nobel prize in 2018. But what is immunotherapy? Immunotherapy is based on recruiting the human body’s own defense mechanisms known as the immune system, to battle cancer. Our immune system is complex and consists of several lines of defense where T cells are the soldiers of the immune system, capable of killing cancer cells. Adoptive cell transfer is a type of immunotherapy in which patients receive new immune cells, most commonly T cells to fight cancer. This thesis is focused on equipping T cells with chimeric antigen receptors (CARs) to efficiently target and kill cancer cells upon adoptive cell transfer. For this the patient’s own T cells are isolated, activated and engineered to express a CAR that can recognize molecules expressed by cancer cells in the laboratory. Following expansion of the CAR T cells, they are infused back into the patient where they can now kill cancer cells upon recognizing them. Targeting the CD19 molecule in blood cancers with CAR T cells has cured many patients.

The overall aim of the thesis was to evaluate the potential of targeting the MSLN molecule with CAR T cells for the treatment of ovarian cancer. The design of the CAR construct can influence their killing potential. In **paper I** and **II** we compared two MSLN-CAR designs containing either a CD28 (M28z) or 4-1BB (MBBz) signaling molecule and T cell activation domain. The CD28 and 4-1BB molecules are naturally occurring receptors involved in stimulating T cell activation. We demonstrated that both M28z and MBBz CAR T cells were capable of successfully killing ovarian tumor spheroids in a laboratory setting (*in vitro*) and prolonged survival of mice with ovarian cancer (*in vivo* models). M28z CAR T cells were able to kill tumor cells more rapidly than MBBz CAR T cells, however, MBBz CAR T cell treatment resulted in long-term tumor control in some mice.

In **paper III**, a new MSLN-CAR design named M1xx was included. The new CAR contained a CD28 molecule and targeted mutations in the T cell activation domain, these mutations result in dampened T cell activation as compared to the ‘classical’ M28z and MBBz CAR designs<sup>4</sup>. The dampening of T cell activation in M1xx CAR T cells resulted in remarkable tumor clearance and long-term disease control in mice with solid ovarian tumors. In a more complex

mouse model of ovarian cancer representing metastasized disease, M1xx CAR T cells were not able to induce long-term tumor control and were only capable of delaying tumor growth.

We identified several mechanisms through which the tumor cells escaped being killed by MSLN-CAR T cells. One of these mechanisms is to reduce presence of the MSLN molecule on tumor cell surface, so CAR T cells are unable to recognize them. Another mechanism was the nibbling of tumor cells by MSLN-CAR T cells. Through this nibbling, the tumor cells lost the MSLN molecule and the MSLN-CAR T cells gained the MSLN molecule which turned them into a target for other MSLN-CAR T cells. Ovarian cancer poses a challenging environment for MSLN-CAR T cell therapy and being challenged in this environment for longer periods leads to exhaustion of MSLN-CAR T cells. Exhaustion is a gradual process in which T cells end up losing their killing capacity.

If we put the fight against ovarian cancer in the context of running a race with the first milestone being to reach the 10 km, the second to finish the half marathon and lastly to crush a full marathon, which advantage do the different MSLN-CAR T cell constructs give patients in running their race? M28z and MBBz CAR T cells are capable of pushing patients through the first 10 km and in rare cases, MBBz-treated patients can finish a half marathon. Treatment with M1xx CAR T cells drives patients past the half marathon milestone. However, M1xx CAR T cell treatment alone is not enough for patients to successfully finish the full marathon of advanced and metastasized ovarian cancer. The future for successful treatment of ovarian cancer with MSLN-CAR T cells, lies in combining MSLN-CAR T cell therapy with other therapies targeting escape mechanisms so that ovarian cancer patients can finish their full marathon.

## ABSTRACT

Chimeric antigen receptor (CAR) T cells have revolutionized the field of immunotherapy, by redirecting T cell specificity and effector functions. Co-stimulation has proven to be crucial for therapeutic effectiveness of CAR T cells and remarkable clinical response rates have been achieved with second generation CD19-directed CAR T cells containing either a CD28 or 4-1BB co-stimulatory domain for the treatment of B cell malignancies. Mesothelin (MSLN) has emerged as an attractive target for CAR T cell therapy in solid tumors, including ovarian cancer. Due to the complex tumor microenvironment niche of ovarian cancer, it is crucial to investigate the mechanisms impacting CAR T cell functionality to improve therapeutic effectiveness. The aim of this thesis was to evaluate the therapeutic potential of three different second generation MSLN-directed CAR T cells for the treatment of ovarian cancer.

Selection of the most advantageous co-stimulatory segment for functional MSLN-CAR T cell persistence in the relevant disease setting is of great importance for successful clinical application. In **paper I** and **II**, MSLN-directed CAR T cells containing either a CD28 (M28z) or 4-1BB (MBBz) co-stimulatory domain followed by the CD3 $\zeta$  chain were compared in different models of ovarian cancer. M28z and MBBz CAR T cells elicited powerful anti-tumor responses in *in vitro* and preclinical *in vivo* models of ovarian cancer, although with different kinetics. Introduction of a CD28 co-stimulatory domain facilitated rapid activation of effector functions, while 4-1BB co-stimulation favored functional persistence of MSLN-CAR T cells.

In **paper III**, a new MSLN-CAR construct was included and evaluated with respect to the classical second generation CAR constructs M28z and MBBz. The novel construct (M1xx) had calibrated activation potential through mutations in the CD3 $\zeta$  chain combined with CD28 co-stimulation. M1xx CAR T cells displayed superior tumor control as compared to M28z and MBBz CAR T cells in two different *in vivo* models. Treatment with M1xx CAR T cells resulted in tumor clearance and long-term remission in the orthotopic mice model. However, in a disseminated disease model M1xx CAR T cells treatment delayed tumor progression substantially but mice eventually succumbed to tumor burden.

In all papers, I attempted to elucidate the mechanisms affecting MSLN-CAR T cell functionality and several immune escape pathways were highlighted. Tumor cells were capable of evading immune control by downregulation of MSLN surface expression and upregulation of the PD-L1 and HLA-DR co-inhibitory ligands on the remaining MSLN<sup>+</sup> tumor cells. Functional persistence of CAR T cells was limited due to exhaustion of MSLN-CAR T cells *in vivo*. Moreover, MSLN-CAR T cells displayed trogocytotic capacity thereby facilitating fratricide killing as well as tumor antigen escape.

## LIST OF SCIENTIFIC PAPERS

- I. **Schoutrop E**, Renken S, Micallef Nilsson I, Hahn P, Poiret T, Kiessling R, Wickström SL, Mattsson J, Magalhaes I. Trogocytosis and fratricide killing impede MSLN-directed CAR T cell functionality. *Oncoimmunology*. 2022 Jun 28;11(1):2093426. DOI: 10.1080/2162402X.2022.2093426.
- II. **Schoutrop E\***, El-Serafi I\*, Poiret T\*, Zhao Y, Gultekin O, He R, Moyano-Galceran L, Carlson JW, Lehti K, Hassan M\*\*, Magalhaes I\*\*, Mattsson J\*\*. Mesothelin-Specific CAR T Cells Target Ovarian Cancer. *Cancer Res*. 2021 Jun 1;81(11):3022-3035. DOI: 10.1158/0008-5472.CAN-20-2701.
- III. **Schoutrop E\***, Poiret T\*, El-Serafi I\*, Zhao Y, He R, Moter A, Henriksson J, Hassan M, Magalhaes I\*\*, Mattsson J\*\*. Tuned activation of MSLN-CAR T cells induces superior anti-tumor responses in ovarian cancer models. *In manuscript*.

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## SCIENTIFIC PAPERS NOT INCLUDED IN THIS THESIS

Foord E, Klynning C, **Schoutrop E**, Förster JM, Krieg J, Mörtberg A, Müller MR, Herzog C, Schiegg D, Villemagne D, Fiedler U, Snell D, Kebble B, Mattsson J, Levitsky V, Uhlin M. Profound Functional Suppression of Tumor-Infiltrating T-Cells in Ovarian Cancer Patients Can Be Reversed Using PD-1-Blocking Antibodies or DARPin® Proteins. *J Immunol Res*. 2020 Aug 4;2020:7375947. DOI: 10.1155/2020/7375947.

**Schoutrop E\***, Moyano-Galceran L\*, Lheureux S, Mattsson J, Lehti K, Dahlstrand H, Magalhaes I. Molecular, cellular and systemic aspects of epithelial ovarian cancer and its tumor microenvironment. *Semin Cancer Biol*. 2022 Apr 5:S1044-579X(22)00083-9. DOI: 10.1016/j.semcancer.2022.03.027.

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## LIST OF ABBREVIATIONS

ACT	Adoptive cell therapy
APC	Antigen presenting cell
ATP	Adenosine 5'-triphosphate
CAF	Cancer-associated fibroblast
CAR	Chimeric antigen receptor
CIM	Co-inhibitory molecule
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
ECM	Extracellular matrix
FAO	Fatty acid oxidation
HGSC	High-grade serous cancer
HLA	Human leukocyte antigen
ICB	Immune checkpoint blockade
IL-2	Interleukin-2
IFN	Interferon
ITAM	Immunoreceptor tyrosine-based activation motif
FACS	Fluorescence-Activated Cell sorting
LAG-3	Lymphocyte activation gene-3
M1xx	MSLN-CD28-1xx
M28z	MSLN-CD28-CD3 $\zeta$
MBBz	MSLN-4-1BB-CD3 $\zeta$
MHC	Major histocompatibility complex
MSLN	Mesothelin
NSG	NOD scid gamma
OXPHOS	Oxidative phosphorylation
PD-1	Programmed cell death-1
TAA	Tumor-associated antigen
TAM	Tumor-associated macrophage
TCR	T cell receptor

Th	T helper
TIL	Tumor infiltrating lymphocyte
TIM-3	T cell immunoglobulin and mucin domain 3
TME	Tumor microenvironment
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TSA	Tumor-specific antigen
UT	Untransduced T cells
VEGF	Vascular endothelial growth factor

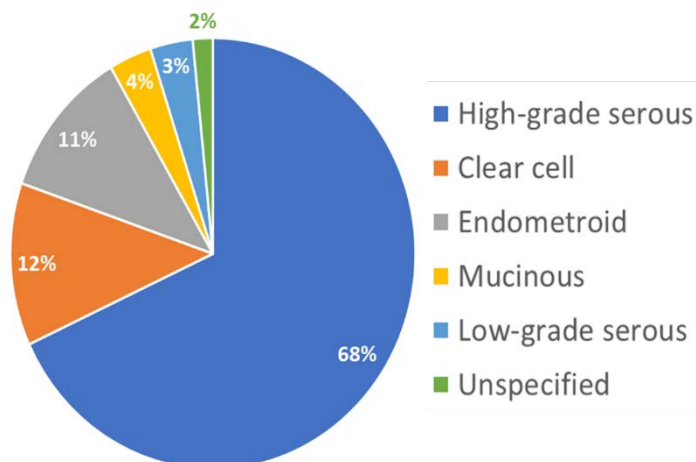
# 1 INTRODUCTION

## 1.1 OVARIAN CANCER

Cancer is an ancient disease which has been studied throughout history, with the earliest report of cancer dating back to 3000 BC in Ancient Egypt<sup>5</sup>. The study of cancer is referred to as oncology and major advances have been made within the field of oncology in the past decades. Despite these efforts, cancer takes the lives of approximately 10 million people across the globe per year<sup>6</sup>. The challenge in finding a cure for cancer lies within the origin of the disease, as it is the result of a cascade of malfunctions within one's own cells – and how do we eliminate these cancerous cells without killing one's self?

Ovarian cancer is the most lethal gynecological malignancy and ranks 8<sup>th</sup> in cancer-related deaths among women worldwide, underlining the urgent need for curative treatment options<sup>6</sup>. Regardless of advancements made within the field of cancer therapy, overall survival rates for ovarian cancer have not substantially improved in the past 25 years<sup>7</sup>. One major obstacle in the fight against ovarian cancer, is the late stage of disease detection as the majority of patients are diagnosed at an advanced disease stage (FIGO stage IIB-IV)<sup>1 7 8</sup>. Advanced ovarian cancer is characterized by widespread disease dissemination within the abdomen and beyond. Survival rates differ between countries and globally the 5-year survival rate for advanced ovarian cancer ranges around 30%, whereas 10-year survival rates of approximately 16% have been reported<sup>2 7-9</sup>.

The vast majority of ovarian tumors (90%) are of epithelial origin, while a small fraction of ovarian tumors originates from germ cells or sex-cord-stromal tissues<sup>1</sup>. Ovarian cancer is an umbrella term describing distinct malignancies originating from the ovaries, fallopian tubes or peritoneum. The origin and multistep development of ovarian cancer have been subject of debate for years<sup>10</sup>. Not only the tissue of origin but also the cell of origin adds to the complexity and heterogeneity of ovarian cancer. Ovarian cancer can be classified into various subtypes or histology types (histotypes) (**Figure 1**), of which high-grade serous cancer (HGSC) is the most common histotype. HGSC accounts for the vast majority of ovarian cancer-related fatalities, which can be attributed to the extremely aggressive nature of HGSC and the late disease detection, as 80% of the patients are diagnosed with FIGO stage III-IV disease<sup>11 12</sup>. In the last



two decades, evidence of an extra-ovarian origin of HGSC arose, these studies identified serous tubal intraepithelial carcinomas (STICs) found within the fallopian tubes as precursor lesions for the majority of HGSC cases.

**Figure 1.** Proportion of most common ovarian cancer histotypes. Adapted from Schoutrop 2022<sup>13</sup>.

What is important to keep in mind, having a STIC as a precursor lesion effectively means that the ovarian tumor is not primary and the disease has already spread from the fallopian tubes to the surrounding tissues<sup>14-16</sup>.

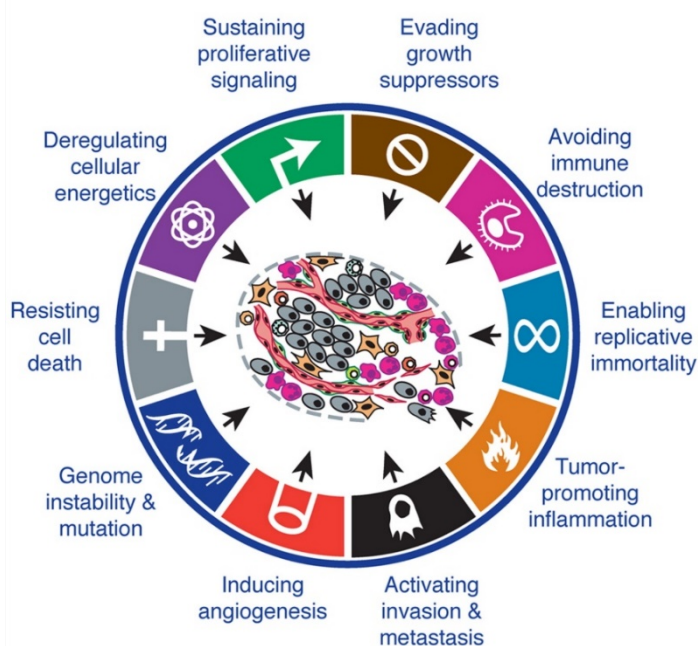
Besides STICs, cortical inclusions cysts (CICs) which develop from invaginations of ovarian surface epithelium (OSE) have been identified as precursor lesions for HGSC in certain patients<sup>17 18</sup>. The dual origin of HGSC, being either fallopian or OSE was confirmed more recently by advanced transcriptomic and proteomic analysis of HGSC patient samples<sup>19 20</sup>. Despite sharing a similar histotype, HGSC and low-grade serous cancer (LGSC) are not to be confused and are classified as two separate disease entities due to their distinct molecular signatures, mechanisms of disease evolution and presumable different cellular origins<sup>11 12 21</sup>.

The major route for ovarian cancer metastasis is transcoelomic and the classical routes of cancer metastasis, in which cancer cells break free from the primary tumor environment and travel to distant locations through the lymphatic and vascular circulation, are of less importance for HGSC disease spread. Ovarian tumor cells optimize their anatomic position for disease spread, with the ovaries freely protruding in the peritoneal fluid without physical border, the tumor cells can passively dissociate from the primary tumor into the peritoneal fluid. During transcoelomic metastasis, there is a buildup of fluid containing freely floating tumor cells in the peritoneal cavity, referred to as malignant ascites. Ovarian cancer cells in the malignant ascites are driven towards the peritoneal surfaces (especially the omentum), where they form micrometastases and can subsequently further infiltrate the abdomen. The widespread disease dissemination in the shape of malignant ascites and micrometastases across the abdomen complicates successful treatment of advanced ovarian cancer<sup>13</sup>. Please keep in mind that cancer metastasis is a complex multistep process and the representation of transcoelomic ovarian cancer dissemination described above is simplified.

The current first-line of care for ovarian cancer patients consists of tumor debulking through extensive surgery complemented with platinum-based chemotherapy, in the presence or absence of the antiangiogenic drug, Bevacizumab<sup>22</sup>. Recently, Poly-ADP-ribose polymerase (PARP) inhibitors (PARPi) have been incorporated as frontline maintenance treatment for HGSC and for recurrent ovarian cancer patients harboring BRCA-1 or BRCA-2 mutations ( $\approx 20\%$ ) or homologous recombination deficient (HRD) tumors, and have shown to prolong progression-free survival<sup>23-25</sup>. Despite therapeutic advancements, the radical treatment regimens and an initial good response to first-line treatment, the majority of patients ( $>70\%$ ) suffers from disease recurrence<sup>3</sup>. There is an urgent need for novel treatment modalities to improve the outcome for ovarian cancer patients, especially for those with advanced ovarian cancer/HGSC currently being failed by conventional therapy.

## 1.2 AVOIDING IMMUNE DESTRUCTION AS A HALLMARK OF CANCER

So what makes healthy cells turn into cancerous cells and how does this result in one or more tumors? The transformation of a healthy cell into a cancer cell, is a complex multistep process in which normal cellular mechanisms are sequentially disrupted. In essence, cancer is a genetic disease that arises by a cascade of mutations, which armor the transformed cells with novel capabilities enabling them to overcome the boundaries of usually tightly regulated cellular processes. Hanahan and Weinberg published their first 'Hallmarks of cancer' paper in 2000, which described six newly acquired capabilities, referred to as hallmarks of cancer, commonly shared between cancer cells regardless of cancer type<sup>26</sup>. The initial six hallmarks of cancer (sustaining proliferative signaling, evading growth suppressors, enabling replicative immortality, activating invasion and metastasis, inducing angiogenesis and resisting cell death)



were supplemented with two new hallmark capabilities and two enabling hallmarks in 2011<sup>27</sup> (**Figure 2**). The new hallmark capabilities are reprogramming cellular metabolism and avoiding immune destruction, the latter being at the core of this thesis. Genomic instability and inflammation are considered enabling hallmarks at the root of the eight hallmark capabilities and driving tumor progression.

**Figure 2.** Hallmarks of cancer as described in 2011 by Hanahan and Weinberg<sup>27</sup>

## 1.3 OUR IMMUNE SYSTEM

The human immune system has an enormous responsibility, constantly scanning the environment for potential threats and subsequently eliminating the threat. We could look at our immune system as an army with several lines of defense. Not only does our immune system protect us from external pathogens, such as bacteria and viruses, but also from internal failures resulting in transformed cells. The latter is referred to as cancer immune surveillance and in case effective, results in the elimination of cancer cells and prevents tumor development<sup>28</sup>.

### 1.3.1 Innate and adaptive immune system: an overview

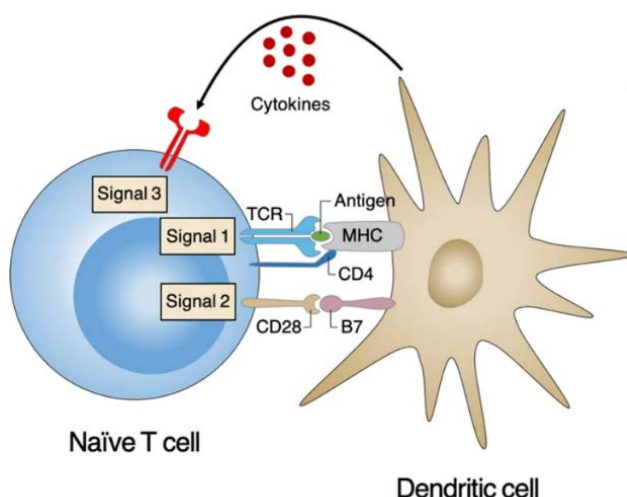
Our immune system is extremely versatile and complex, with the capability to defend us against threats on a daily basis. I will not be able to explain the magic of our full immune system in this thesis, instead, the intent is to provide background context to the research aims within my

PhD project. The immune system can be divided into two arms, known as innate and adaptive immunity. Innate immunity is our first line of defense and can also be referred to as natural immunity since it represents our inborn defense mechanisms, composed of actual physical barriers (the skin), cellular components (macrophages, natural killer (NK) cells and dendritic cells amongst others), and soluble mediators (e.g. cytokines). The main functions of the innate immune system are to provide initial protection against common foreign invaders, wound healing and to recruit suitable adaptive immune responses in more complex situations<sup>29</sup>. Adaptive immune responses are considered more sophisticated than innate responses as they are highly specific and can provide long-lasting protection through the formation of immunological memory. Two types of white blood cells, the T and B lymphocytes mediate adaptive immune responses by signaling through their respective antigen-specific receptors.

The innate and adaptive arms of the immune system work together in the elimination of complex pathogens as well as transformed cells. Dendritic cells, macrophages, NK cells and T cells amongst others play a major part in the recognition and subsequent eradication of cancer cells. Due to the central role of T cells in direct killing of tumor cells, they have been of great interest for the development of immunotherapy and will be explored further in the next section.

### 1.3.2 T cell biology

T cells originate from hematopoietic stem cells in the bone marrow, which migrate to the thymus and here the so-called thymocytes develop into T lymphocytes. During the thymic maturation process, thymocytes undergo selection processes ( $\beta$ -selection, positive and negative selection) based on their unique T cell receptors (TCRs). The cells whose TCRs are capable of recognizing self-antigens bound by major histocompatibility complexes (MHC) complexes I or II with the right affinity (not too strong nor too weak) differentiate into mature CD4 or CD8 T cells<sup>30</sup>. Of note, in the human setting, MHC complexes are referred to as human leukocyte antigen (HLA) complexes. This process results in selection of T cells with a repertoire of functioning TCRs, each specific for one certain antigen-HLA complex. These mature CD4 and CD8 T cell populations are considered naïve as their unique TCRs have never encountered their cognate antigen-HLA complexes on the surface of antigen-presenting cells (APCs). Naïve T cells leave the thymus and travel through the blood and lymphatic system, where they recirculate to browse for cognate antigen with their unique TCR<sup>31-33</sup>.



**Figure 3.** Three signals required for full activation of naïve T cells: antigen stimulation, co-stimulatory signaling and pro-inflammatory cytokines<sup>34</sup>.

Initial activation of naïve T cells requires priming by professional APCs, such as dendritic cells, B cells and/or macrophages, as they are capable of providing co-stimulatory signals which nonprofessional APCs (e.g. cancer cells) cannot. Three signals are required for activation of naïve T cells and development of effector functions (**Figure 3**)<sup>34</sup>:

- 1) Recognition of antigen-HLA complexes by the TCR.
- 2) Co-stimulatory signals by interaction between co-stimulatory molecules present on T cells and APCs, such as the CD28 molecule present on T cells and CD80/CD86 on APCs.
- 3) Secretion of pro-inflammatory cytokines such as IL-12 and IFN $\gamma$  by APCs mediates full activation of T cell effector functions.

**Figure 3.** Three signals required for full activation of naïve T cells: antigen stimulation, co-stimulatory signaling and pro-inflammatory cytokines<sup>34</sup>.

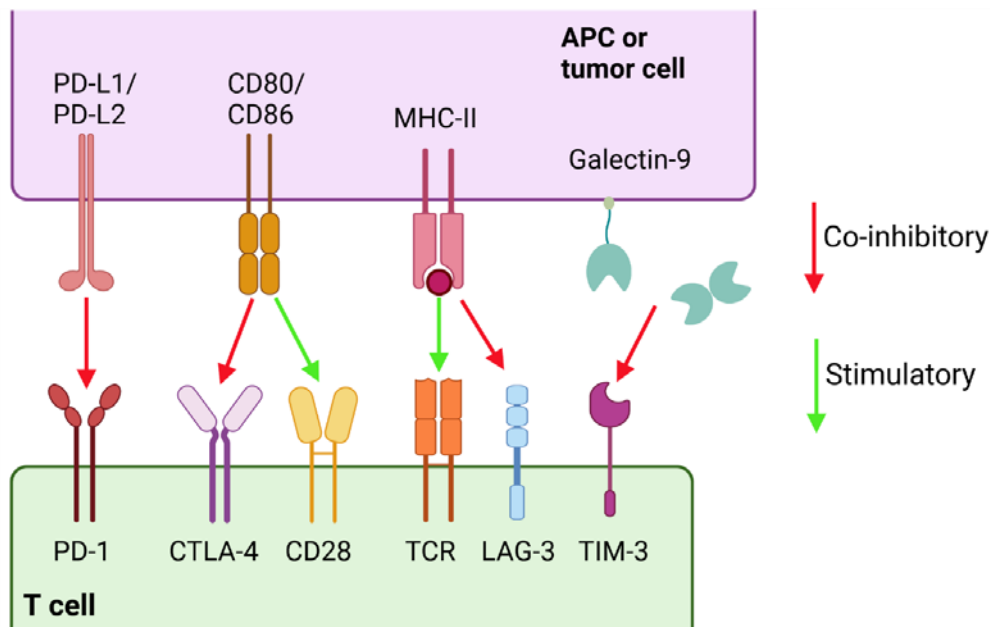
Following activation, the T cells proliferate (clonal expansion) and differentiate into memory or effector T cells. Effector T cells travel to the site of inflammation, where they directly target cognate antigen-HLA complexes and exert their effector functions. CD4 and CD8 T cell populations harbor different effector functions, CD4 T cells differentiate into distinct helper T cell subsets (Th) while naïve CD8 populations differentiate into cytotoxic T lymphocytes (CTLs)<sup>31-33 35</sup>.

The nomenclature of ‘helper’ T cells stems from the fact that CD4 T cells do not directly kill target cells themselves, instead they provide signals to cells of the innate and adaptive immune system, thereby shaping the immune response. During activation by APCs, CD4 T cells receive polarizing signals that drive their differentiation into one of over five helper subsets. Each subset plays a distinct role in the immune system through production of their own signature effector cytokines, driving immune responses in a specific direction. T helper subset 1 (Th1) cells are well known for their ability to promote immunity against intracellular pathogens and tumor cells as they provide the required help for CD8 T cell differentiation into CTLs, amongst others. The CD4 helper subset 2 (Th2) cells are involved in protection against extracellular pathogens and promote B cell activation<sup>31 36</sup>. The balance between Th1 and Th2 helper cells in disease has been of major focus as it influences disease outcome. In cancer, Th1 dominance is associated with favorable prognosis. Another major CD4 subset, regulatory T cells (Treg), are of key importance in limiting immune responses and maintaining immunological tolerance under physiological conditions. However, due to their dampening effect on the immune response, Tregs actively promote cancer progression<sup>37 38</sup>.

Following differentiation of naïve CD8 T cells into CTLs, the CD8 T cells acquire the capability to directly kill target cells, which can be either infected or transformed cells. There are two main mechanisms through which CTLs kill their target cells: 1) the release of toxic

granules containing perforin and granzyme at the immunological synapse and 2) apoptotic signaling through engagement of the FAS death receptor on target cells with FAS ligand expressed by CTLs<sup>31</sup>. Another mechanism through which various effector cells, including CTLs and T helper subset 1 cells, can induce killing of target cells is the production of tumor necrosis factor (TNF) and interferon gamma (IFN- $\gamma$ ) which upon binding to their respective receptors induce apoptosis<sup>39</sup>. CD8 T cells are potent mediators of anti-tumor immunity and presence of CD8 T cells in the tumor microenvironment (TME) of several cancers, including ovarian cancer, has been linked to favorable disease outcome<sup>38</sup>.

In order to maintain self-tolerance and prevent immune hyper-activation, the adaptive immune response needs to be controlled<sup>40 41</sup>. Tregs are key regulators of the immune response and can limit T cell activation through several mechanisms. Moreover, T cell activation triggers the expression of co-inhibitory receptors, such as PD-1, CTLA-4, LAG-3 and TIM-3. Co-inhibitory receptors are the physiological brakes on T cell mediated immune responses as binding to their respective co-inhibitory ligands expressed by APCs terminates the immune response. Due to their regulatory impact on immune responses, co-inhibitory receptors are also referred to as immune checkpoints. Many co-inhibitory molecules (CIMs) have been identified in the past decades and there are undoubtedly more to be discovered. The balance between co-stimulatory and co-inhibitory signals determines the fate of the T cell response, either activation or inhibition (**Figure 4**)<sup>40 41</sup>. Tumor cells have a clever way of employing this physiological braking system to their advantage by upregulating the expression of co-inhibitory ligands under immune pressure to evade killing by T cells<sup>42 43</sup>.

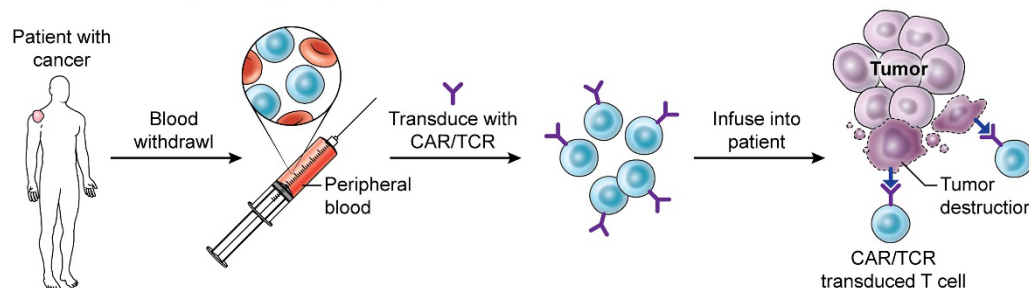


**Figure 4.** Selection of co-stimulatory and co-inhibitory receptors expressed by T cells and their respective ligands expressed by APCs or tumor cells.

## 1.4 ERA OF IMMUNOTHERAPY

### 1.4.1 Introduction to immunotherapy

In the last decades, employment of the immune system in the fight against cancer has been proven to be extremely valuable and several successful immunotherapies have emerged. Immunotherapy has led to a paradigm shift within the field of modern cancer treatment, especially for certain solid tumors including melanoma as well as hematological malignancies. Currently there is a major focus on immune checkpoint blockade (ICB) and adoptive cell transfer (ACT) for the treatment of ovarian cancer<sup>44-45</sup>. The principle of ACT is based on the isolation of autologous or allogeneic lymphocytes, followed by *in vitro* stimulation, modulation and expansion, after which the activated lymphocyte product is infused into the patient (**Figure 5**). The infusion product consists of either tumor-infiltrating lymphocytes or genetically engineered T cells targeting tumor antigens.



**Figure 5.** The process of ACT using CAR T cells starts with the collection of peripheral blood from patients. T cells are isolated from the blood and transduced with a CAR construct, resulting in the expression of the CAR on the T cell surface. These T cells are expanded further prior to re-infusion into the patient, where they can recognize tumor cells through their CAR<sup>46</sup>.

### 1.4.2 Immune checkpoint blockade

Due to the critical role of CIMs in controlling T cell responses and the exploitation of these co-inhibitory pathways by tumor cells to avoid immune destruction, there has been a great interest in circumventing these brakes on the immune system for cancer therapy. This led to the development of immune checkpoint blockade (ICB), which blocks the interaction between co-inhibitory receptors and their respective ligands. ICB inhibits the negative regulatory downstream signaling in T cells and thereby shifts the balance towards T cell activation. Checkpoint blockade targeting the PD-1 and CTLA-4 pathways have led to major clinical successes in melanoma and several other tumors (e.g. Hodgkin lymphoma, lung cancer and microsatellite instable tumors)<sup>47</sup>. Despite the advances made with ICB in various malignancies, checkpoint blockade of PD-1 and CTLA-4 has so far only led to marginal responses in ovarian cancer, ranging from 6-15%<sup>48-51</sup>. Removal of the brakes on the immune system by ICB does not seem to be sufficient for the majority of ovarian cancer patients, but why? One of the major reasons for the marginal response rates with ICI, is the limited presence of tumor-reactive T cells in ovarian cancer<sup>52</sup>. So alleviating the brakes will not result in tumor-directed immune

response in the majority of ovarian cancer patients. Another hurdle is the simultaneous expression of several CIMs, single blockade of PD-1 or CTLA-4 might therefore not be sufficient as alternative co-inhibitory pathways are employed. Combinatorial approaches targeting PD-1/CTLA-4 together with alternative CIMs such as LAG-3 are currently under evaluation for the treatment of ovarian (NCT04611126).

### **1.4.3 Tumor infiltrating lymphocytes (TILs)**

The potential of T cells in the eradication of tumors in humans has been shown repeatedly through ACT of tumor-infiltrating lymphocytes (TILs) since the pioneering work of Rosenberg *et al* in 1988<sup>53</sup>. Clinical application of TIL therapy in metastatic melanoma has resulted in complete remissions in approximately 25% of the patients, demonstrating the curative potential of ACT using TILs<sup>54</sup>. The presence of TILs in the tumor stroma has been positively associated with prognosis in several solid tumors including ovarian cancer. The five-year survival rate for advanced stage ovarian cancer (or HGSC) patients harboring TILs in the tumor stroma was 38%, while five-year survival rate was merely 4,5% in patients devoid of intratumoral T cells<sup>38</sup><sup>55</sup>. Despite the impressive clinical responses in metastatic melanoma, the therapeutic effect of ACT-TIL remains to be proven for the treatment of ovarian cancer<sup>56</sup>. Similar to the limited therapeutic effects of ICB in ovarian cancer, the mere presence of TILs is not a guarantee for successful anti-tumor responses, which are amongst others dependent on the frequency of tumor-reactive TILs in the infusion product and unfortunately, the frequency of tumor-reactive TILs in ovarian cancer is low<sup>52</sup>. Furthermore, the acquisition of immune evasion mechanisms such as the down-regulation of HLA class I by tumor cells, CTLA-4 expression on TILs outcompeting CD28 co-stimulation and the exhausted phenotype of TILs form major hurdles for persistent TIL mediated anti-tumor responses<sup>41 57 58</sup>.

## **1.5 EVOLUTION OF CHIMERIC ANTIGEN RECEPTOR T CELLS**

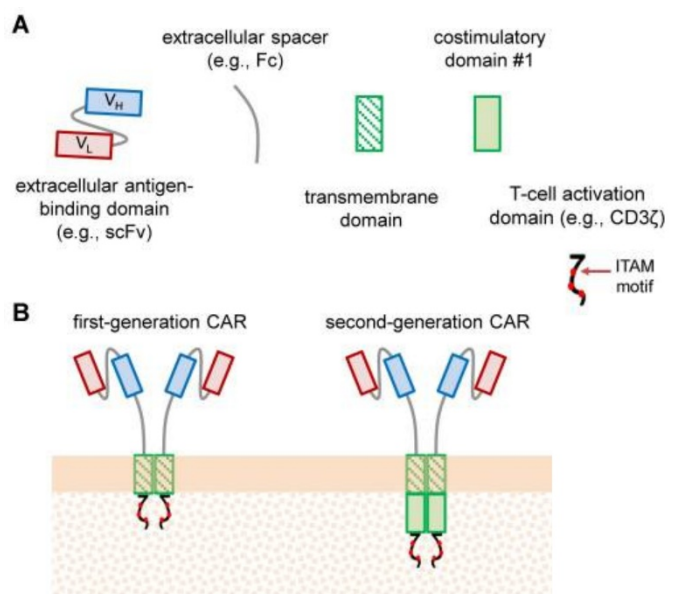
### **1.5.1 History of CAR T cells**

To overcome the biological limitations encountered with ICB and TIL therapy such as the low frequency of tumor-reactive T cells and HLA-I downregulation, ACT with chimeric antigen receptor (CAR) T cells is a promising treatment option. CARs reprogram T cell specificity and function through their engineered structure, which is composed of an extracellular antigen binding domain (scFv), a transmembrane linker domain and an intracellular activation domain. The extracellular domain is derived from the light and heavy chain regions of a monoclonal antibody, enabling the specific binding of surface antigens in a HLA-independent manner. The ability to directly target surface antigen enables CAR T cells to bypass TCR-mediated targeting of antigen-HLA complexes, thereby CAR T cells can overcome HLA downregulation by tumor cells and defects in antigen processing machinery of tumor cells. The intracellular signaling domain almost always contains a CD3 $\zeta$  segment, allowing for activation of T cell effector functions<sup>58 59</sup>.

Pioneering work in the early 90s led to the development of chimeric T cell activating receptors, which resulted in the development of T-bodies, later referred to as first generation CAR T cells<sup>60 61</sup>. The first generation CAR constructs were composed of an extracellular scFv connected to an intracellular CD3 $\zeta$  signaling domain. First generation CAR T cells demonstrated effective tumor cell targeting *in vitro*, however, marginal anti-tumor responses with limited persistence were observed in mice models<sup>62 63</sup>. Similar results were observed in phase I studies using first generation CAR T cells for the treatment of ovarian cancer, renal cancer or neuroblastoma, resulting in minimal clinical response rates<sup>64-66</sup>.

Extensive efforts within the field led to the development of second generation CAR T cells in the early 2000s. The structure of 2<sup>nd</sup> generation CAR constructs was changed by incorporation of a co-stimulatory molecule upstream of the CD3 $\zeta$  chain as can be seen in **Figure 6**. The introduction of a co-stimulatory domain boosted CAR T cell expansion, activation, anti-tumor efficacy and persistence<sup>67 68</sup>. Various co-stimulatory domains are currently being used, such as ICOS, CD28, 4-1BB, OX40 and more. However, CD28 and 4-1BB are the most widely used and to date, the only co-stimulatory domains approved for clinical application. Treatment with second generation CD19-directed CAR T cells incorporating a CD28 or 4-1BB domain have led to remarkable complete remissions in patients with refractory CD19+ hematological malignancies<sup>69</sup>. The big clinical successes booked with CD19-directed CAR therapy have led to FDA and EMA approval of four different CD19-CAR T cell products since 2017, containing either CD28 or 4-1BB co-stimulation (tisagenlecleucel, axicabtagene ciloleucel, brexucabtagene autoleucel, and lisocabtagene maraleucel)<sup>70-73</sup>.

**Figure 6.** Design of first and second generation CAR T cells. **A)** Separate constituents of CAR constructs. **B)** Structure of CAR constructs composed of a monoclonal antibody derived extracellular domain, the scFv region which is connected to the intracellular T cells signaling domains through an extracellular spacer/hinge region and transmembrane domain. Adapted from Chen *et al*, Molecular medicine 2017<sup>74</sup>.



### 1.5.2 CD28 versus 4-1BB co-stimulation

The incorporation of a co-stimulatory domain, was without a doubt a game changer for CAR T cell application. As previously discussed, TCR signaling and co-stimulation are required for T cell activation. Through the engineered structure of second generation CAR T cells, co-stimulation and activating T cell signaling are provided at once upon binding of the target antigen. Due to this, second generation CAR T cells, containing either a CD28, 4-1BB or other co-stimulatory domain are superior to first generation CAR T cells. The CD28 co-stimulatory domain naturally occurs on resting and activated T cells as a homodimer and provides co-

stimulatory signaling upon binding CD80/CD86<sup>75</sup>. Expression of 4-1BB is upregulated upon T cell activation or antigen stimulation. Once expressed at the cell surface, 4-1BB occurs as a monomer or dimer and provides co-stimulatory downstream signaling upon binding the 4-1BB ligand (4-1BBL)<sup>76 77</sup>. Co-stimulatory signaling through either CD28 or 4-1BB promotes T cell activation, survival and proliferation.

The impact of CD28 and 4-1BB co-stimulation on CAR T cell functionality has been widely studied in preclinical models in the last two decades. The majority of studies have been focused on hematological malignancies, however research regarding CAR T cell application for solid tumors has seen an upsurge. Preclinical mice models of hematological malignancies have reported contradicting outcomes, as in some studies CD28 co-stimulation was superior<sup>78 79</sup>, sometimes no differences were detected between CD28 and 4-1BB co-stimulatory domains<sup>80</sup>, whereas 4-1BB co-stimulation was shown to be superior by others studies<sup>81-83</sup>. No direct comparison has been made between CD28 and 4-1BB co-stimulated CD19-CAR T cells in a large cohort clinical trial, and the currently available results from clinical studies demonstrate comparable therapeutic effectiveness between CD28 or 4-1BB co-stimulated CD19-CAR T cell therapy for the treatment of B cell malignancies<sup>71 72 84 85</sup>. Moving beyond CD19-CAR T cells, the majority of preclinical *in vivo* solid tumor models reported either superior or comparable tumor control by 4-1BB co-stimulated CAR T cells as compared to CAR T cells containing a CD28 co-stimulatory domain. Several preclinical studies have demonstrated enhanced persistence and decreased levels of exhaustion by 4-1BB co-stimulated CAR T cells, presumably attributing to their superior functional persistence<sup>86-90</sup>.

CD28 and 4-1BB co-stimulatory domains employ distinct signaling and metabolic pathways, which attribute to differences in functional persistence and exhaustive state. CD28 co-stimulation results in rapid activation of the P13K-Akt pathway amongst others, which impacts glucose metabolism, resulting in increased glycolysis. Ligation of the 4-1BB co-stimulatory domain, invokes different signaling pathways through recruitment of TNFR-associated factors (TRAFs), and fatty acid oxidation (FAO) is the preferred metabolic pathway of 4-1BB co-stimulated CAR T cells<sup>91 92</sup>. In the solid TME, usage of the most beneficial metabolic pathway (glucose versus oxidative metabolism) is crucial for functional persistence. To shine a light on this matter, a brief overview on the usage of metabolic pathways by T and tumor cells is provided below. However, cellular metabolism is complex and due to the scope of this thesis, I will not be able to go in depth on this subject. Quiescent and activated T cells have a different metabolic state: resting T cells use OXPHOS and FAO for glucose conversion into energy (ATP) while activated T cells increase the glycolysis pathway over OXPHOS and FAO. Aerobic glycolysis utilized by activated T cells is required for proliferation, survival and IFN $\gamma$  production<sup>93</sup>. In the TME there is competition between cancer cells and TILs for glucose uptake and subsequently the uptake of glucose by cancer cells leads to nutrient restriction for the TILs which in turn leads to cytokine repression. The expression of glucose transporter 1 (Glut1) on cells depends on glucose availability and hence is ultimately downregulated on T cells within the TME, creating a negative feedback loop<sup>93 94</sup>.

One take home message from this is that superior activity by either CD28 or 4-1BB co-stimulated CAR T cells depends on the specific conditions of each model. Due to the complexity of the solid tumor microenvironment, identifying the most suitable CAR construct leading to long-term functional persistence in the relevant disease setting is crucial for treatment outcome. Other considerations to keep in mind when interpreting results comparing the functionality of CD28 and 4-1BB co-stimulated CAR T cells, are the distinct manufacturing processes between study centers and additional differences in the CAR design, such as the hinge and transmembrane domain.

### **1.5.3 Advancements in CAR design**

Since the introduction of second generation CAR T cells, the field has continued to innovate and introduce new CAR T cell designs. Some strategies ‘armor’ the CAR T cells with new beneficial capabilities while others target the intracellular signaling of CAR T cells.

For instance, to overcome the immunosuppressive cytokine networks in the TME, CAR T cells can be armored with immune-stimulatory cytokines. Interleukin-12 (IL-12) is a booster of T cell activation and effector functions, with the capacity to shift the negative cytokine balance towards a positive balance<sup>95</sup>. An armored MUC-16-directed CAR construct constitutively secreting IL-12 has shown promising superior tumor control and persistence in a preclinical ovarian cancer model. These armored CAR T cells augmented anti-tumor efficacy by modulating the tumor microenvironment via autocrine activity on CAR T-cells, depletion of tumor-associated macrophages (TAMs) and resisting PD-L1 induced inhibition<sup>96 97</sup>. Another novel strategy in overcoming inhibition induced by the PD-1-/PD-L1 axis is incorporation of a PD-1:CD28 switch receptor in the CAR construct. The switch receptor consists of the truncated extracellular domain of PD-1 connected to the CD28 co-stimulatory domain. The switch receptor has a promising effect on the functionality of several different CAR T cells in aggressive preclinical models, including MSLN directed CAR T cells. Introduction of the PD-1:CD28 decreases susceptibility to PD-L1 induced T cell dysfunction and enhances tumor eradication, tumor infiltration by CAR T cells<sup>97</sup>.

The use of CRISPR/Cas9 allows for introduction of the CAR construct at a specific site in the genome and direction of the CD19-CAR construct to the T cell receptor- $\alpha$  constant (TRAC) locus has shown to be beneficial, augmenting T cell potency and delaying T cell exhaustion<sup>98</sup>. More recently, another strategy for extending functional persistence of CAR T cells was developed by prof. Sadelain and colleagues. The traditional CD3 $\zeta$  domain contains three immunoreceptor tyrosine-based activation motifs (ITAMs). Strong T cell activation through CD3 $\zeta$  drives exhaustion, and calibration of ITAM activity through point mutations in the two distal motifs balances effector and memory T cell functions. CD28 co-stimulated CD19-CAR T cells with a single functioning proximal ITAM motif displayed superior anti-tumor potency, persistence and minimal functional exhaustion as compared to their classical second generation counterparts in a preclinical model<sup>4</sup>. Applying novel strategies in CAR T cell design for the treatment of solid tumors is a promising tool for overcoming obstacles imposed by the TME and enhancing therapeutic effectiveness.

### 1.5.4 Moving beyond CD19-directed CAR T cells

One of the major bottlenecks for successful translation of CD19-CAR T cell therapy to solid tumors such as ovarian cancer is the identification of suitable tumor antigen targets. The perfect antigen is homogeneously high expressed in tumor cells and absent in healthy tissues. Tumor antigens can be broadly classified as tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs). TSAs originate from gene mutations during cancer development and result in the expression of neoepitopes by tumor cells. Therefore, TSAs are high-specificity antigens and are thus not shared between patients, requiring individualized immunotherapy. TAAs and cancer testis antigens are shared between specific patient groups and are thus a potential target for CAR T cell therapy<sup>99</sup>. TAAs are normal peptides displaying a different expression profile between healthy tissues and cancer cells, they can be further classified as differentiation antigens and overexpressed antigens. Differentiation antigens are expressed in a certain tumor type and the corresponding healthy cells, such as CD19 on B cell malignancies and healthy B cells. As the name already suggests, overexpressed antigens are overexpressed on malignant cells with low expression levels on normal tissues<sup>100</sup>. One such antigen is mesothelin (MSLN), with limited expression levels on healthy cells and overexpressed in a broad spectrum of solid tumors, including ovarian cancer<sup>101</sup>. Given this expression pattern MSLN is an interesting target to further explore for CAR T cell therapy in ovarian cancer amongst others.

## 1.6 MESOTHELIN ANTIGEN

### 1.6.1 Biology of Mesothelin

In the early 1990s, MSLN was discovered as a potential target for antibody-based therapies in several solid tumors due to its pattern of expression<sup>102</sup>. Despite the discovery of MSLN over 20 years ago, its biological function in humans remains unknown. Under physiological conditions it's a differentiation antigen found on the mesothelial cells lining the peritoneum, pericardium, and pleura. It does not appear to have a critical function in development as was shown in knockout mice models<sup>103</sup>. However, some preclinical and clinical studies have linked MSLN overexpression to tumor transformation and aggressiveness. Although the exact function of MSLN in cancer progression remains to be elucidated and might be cancer-type specific<sup>101</sup>.

MSLN is a glycoprotein attached to the cell membrane by a GPI anchor domain. It is initially synthesized as a 71 kDa cell-surface protein which is cleaved by the protease furin leaving behind a cell membrane bound mesothelin protein (C-terminus) and a soluble fragment, named megakaryotic-potentiating factor (MPF)<sup>104</sup>. During post-translational modifications a glycolipid (GPI) is attached to the C terminus, facilitating anchorage to the cell membrane. GPI-anchored proteins can be released from the cell membrane through cleavage by phospholipases or by proteases. This process is known as shedding and results in the release of soluble mesothelin related peptide (SMRP) in the circulation<sup>101 104 105</sup>. SMRP is generated by cleavage of cell membrane anchored MSLN by the TNF converting enzyme (TACE) protease

and/or by alternative splicing (MSLN variant 3). SMRP levels are significantly elevated in the serum and ascites of ovarian cancer patients<sup>105-107</sup>.

### **1.6.2 Mesothelin in ovarian cancer**

MSLN is overexpressed in the majority of HGSC cases, but how does MSLN overexpression look like in the complex setting of advanced ovarian cancer with malignant ascites and widespread micrometastases across the abdomen? Overexpression of MSLN has been reported in 55% to 97% of serous ovarian tumors, this range can be explained by usage of distinct cut-off values for positivity in different centers. Important to keep in mind, is that once an ovarian tumor is classified as MSLN positive, this does not mean all cancer cells within the primary tumor nor metastases express MSLN<sup>108-110</sup>.

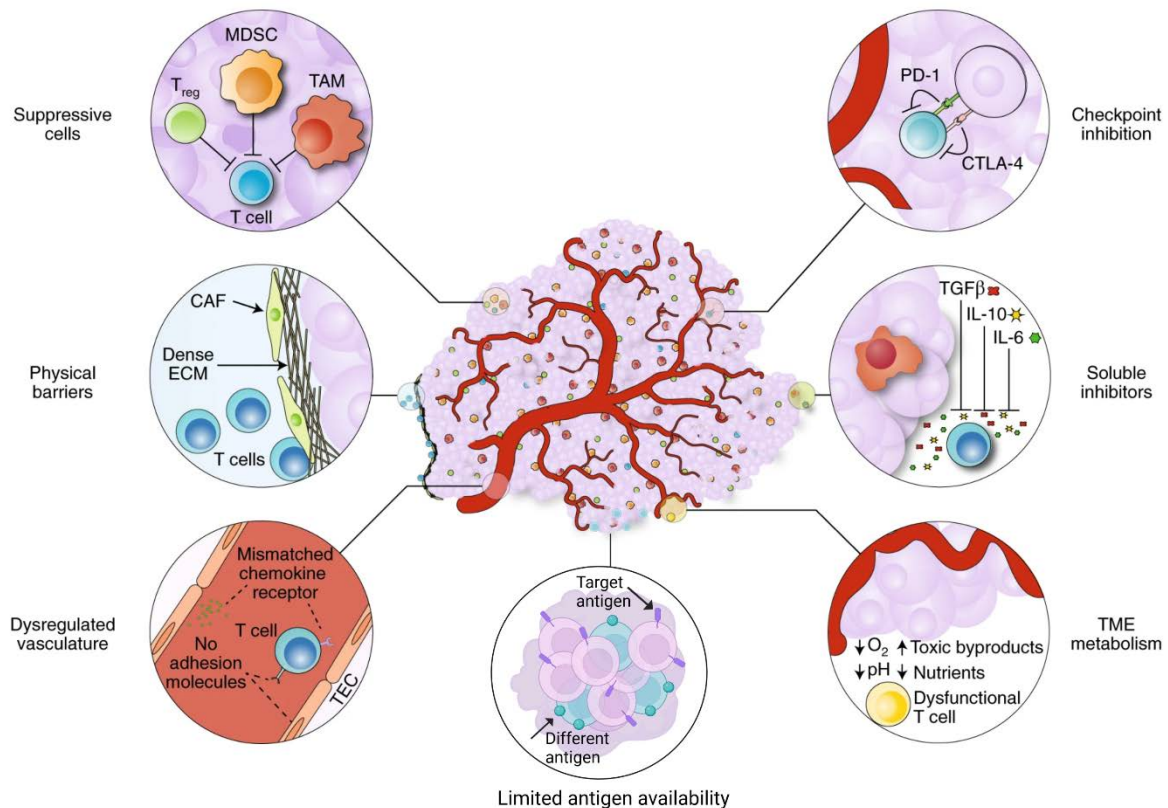
In several solid tumors MSLN has been implicated in cancer proliferation, invasion and metastasis<sup>111</sup>. It is proposed that MSLN promotes ovarian cancer progression through interaction with cancer antigen 125 (CA125, interchangeably referred to as MUC16), which is also overexpressed in ovarian cancer. MUC16 and MSLN can bind each other in a homotypic way (cancer cell to cancer cell), thereby promoting cell detachment from the primary tumor and generation of freely floating cancer spheroids, and in a heterotypic manner (cancer cell to mesothelial cell), therewith supporting adhesion of spheroids to the peritoneal mesothelial layer and subsequent invasion<sup>112 113</sup>. However, the impact of MSLN overexpression on clinical outcome and effective contribution to ovarian cancer dissemination within the abdomen has not been elucidated and to date, contradicting findings have been reported<sup>108 111 114-116</sup>.

## **1.7 COMPLICATIONS FOR CAR THERAPY IN SOLID TUMORS**

### **1.7.1 The fight of CAR T cells in solid tumors**

So far, the unprecedented clinical results achieved with CD19 CAR T cell therapy in hematological malignancies have not been achieved in solid malignancies<sup>117</sup>. MSLN-CAR T cells are currently under investigation in various early stage clinical trials for the treatment of malignant mesothelioma, pancreatic cancer and ovarian cancer amongst others. These studies so far demonstrated a good safety profile for MSLN-CAR T cells, without substantial on-target/off-tumor related toxicities. In the small cohort of ovarian cancer patients treated with MSLN-CAR T cells, the response rates have thus far been disappointing. The best overall response achieved was stable disease and despite tumor reductions in a minority of patients, partial response was not achieved according to the RECIST 1.1 criteria<sup>118-120</sup>. The marginal response rates observed in solid tumors can be attributed to several hurdles encountered in the solid tumor milieu, including limited CAR T cell trafficking and homing to the tumor site, the immunosuppressive TME and limited antigen availability<sup>117</sup>.

An overview of the mechanisms impacting CAR T cell proliferation, persistence and functionality in the solid tumor milieu can be seen in **Figure 7** and will be discussed in more detail in the upcoming sections.



**Figure 7.** Overview of the mechanisms negatively impacting T cell mediated anti-tumor responses in the solid TME niche. Adapted from Labanieh *et al*, Nature Biomedical Engineering, 2018<sup>121</sup>.

### 1.7.2 Trafficking to the tumor

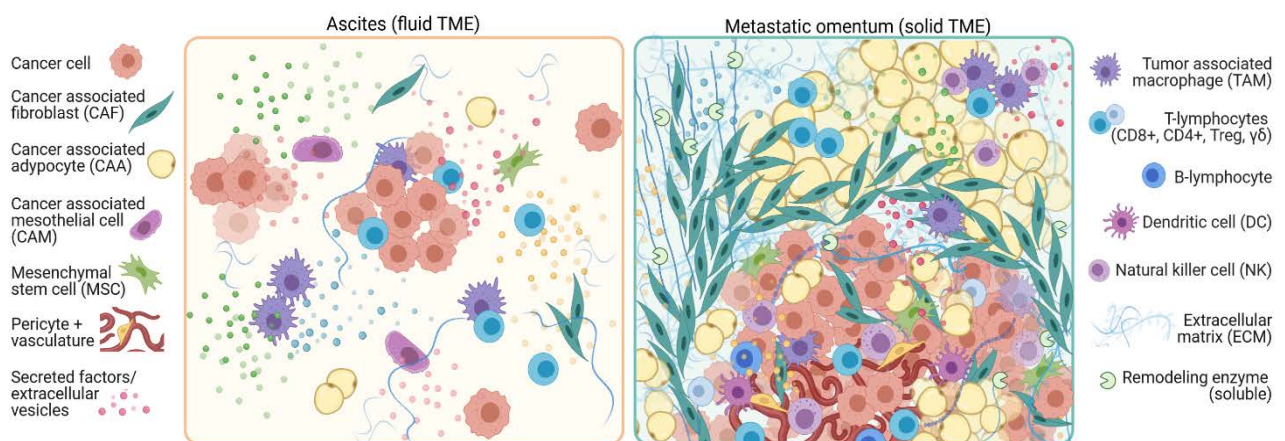
Cancer cells are prevalent within the blood circulation and bone marrow of hematological malignancies, therefore, intravenously administered CD19-CAR T cells have the major advantage of easily accessible tumors cells. The readily available (neoplastic) B cells serve as APCs, providing proliferative and activating signals to the CAR T cells. On the contrary, in solid tumors the CAR T cells first have to home to tumor site through the circulation following intravenous administration<sup>122</sup>. The delayed kinetics in CAR trafficking to the tumor site can potentially be overcome by locally delivered CAR T cells instead of intravenous CAR infusion. Superior tumor control has been demonstrated with locally delivered over intravenously delivered CAR T cells in different solid tumor models (e.g. breast cancer, mesothelioma)<sup>86 123 124</sup>.

The process of leukocyte trafficking and infiltration of peripheral tissues is a complex cascade of events, which is unfortunately dysregulated in tumors. Chemokines and adhesion molecules are responsible for the capture, rolling, adhesion and transmigration of lymphocytes into peripheral tissues<sup>117 122 125 126</sup>. Tumors are characterized by abnormal vasculature and expression of adhesion and extravasation molecules, limiting efficient CAR T cell trafficking.

Furthermore, the chemokine and chemokine-receptor signaling is not always compatible between the tumor sites and effector T cells, further impeding homing of CAR T cells into the tumor<sup>125-127</sup>. To complicate matters further, once the CAR T cells manage to reach the tumor they are subject to the physical barriers and hostile environment imposed by the TME.

### 1.7.3 Tumor microenvironment

The success of immunotherapy in solid tumors has been limited, largely due to the immunosuppressive impact of the tumor microenvironment. The TME of ovarian cancer is unique, encompassing both fluid and solid TME niches due to the transcoelomic disease spread. Malignant ascites embodies the fluid TME, while the metastatic omentum is part of the solid TME. The fluid and solid TME encompass similar non-malignant cellular and acellular factors, although with different frequencies and with the exception of pericytes and endothelial cells being specific for the solid niche. The non-malignant cellular compartment of the TME encompasses a mixture of immune cells (tumor-associated macrophages (TAMs) and lymphocytes amongst others) and stromal cells (cancer-associated fibroblasts (CAFs), cancer-associated mesothelial cells and more). The acellular fraction of the TME is a reservoir of cytokines, growth factors and extracellular matrix (ECM) proteins amongst others<sup>128-130</sup>. An overview of the fluid and solid TME can be seen below in **Figure 8**.



**Figure 8.** Overview of the main compartments of the fluid and solid TME niches of ovarian cancer<sup>13</sup>.

TAMs are amongst the most abundant immune cells found within the ovarian cancer TME and play a pivotal role in promoting disease progression. In malignant ascites, TAMs mediate tumor spheroid formation and drive peritoneal disease dissemination<sup>131</sup>. Similar to CD4 T cell differentiation, polarizing cytokines drive TAM differentiation into a specific subset. Broadly, TAMs can be classified as the pro-inflammatory type 1 (M1) or immunosuppressive type 2 (M2) macrophages. This classification system does not do justice to the heterogeneous population of TAM subsets found *in vivo*<sup>132-134</sup>. It comes as no surprise, that the immunosuppressive HGSC niche promotes M2 polarization, which in-turn drive disease progression and metastasis further through a plethora of immune-modulatory mechanisms<sup>135</sup>

CAFs are a major component of the ovarian cancer TME and have a pivotal role in the initial steps of disease dissemination as well as further disease progression. The frequency of CAFs differs between the solid and fluid TME niche, being abundant within the metastatic omentum and less prevalent within the malignant ascites. Despite different frequencies, CAFs actively promote ovarian cancer progression in both the solid and fluid TME niches<sup>137-139</sup>. Similar to TAMs, CAFs drive initial ovarian cancer progression by mediating tumor spheroid formation within the malignant ascites and promote the subsequent seeding of the peritoneal cavity with tumor spheroids. CAFs produce a variety of soluble factors actively promoting ovarian cancer cell survival and proliferation and invasion of the peritoneal cavity by tumor cells (growth factors such as EGF, chemokines and proteases)<sup>138 139</sup>. Another major mechanism through which CAFs actively promote tumor progression is the excessive production of ECM components. Essentially the ECM is the environment in which cells exist and is a dynamic mixture of insoluble proteins (collagens), proteoglycans and adhesive glycoproteins providing a site for cell anchorage, guiding cell migration and transmission of environmental cues to the cells. In cancer, both CAFs and tumor cells remodel the ECM through degradation and deposition of ECM components creating an environment optimal for cancer cell survival, proliferation and invasion<sup>130 140</sup>. In the metastatic omentum, tumor cells and CAFs drive the transformation of fatty omentum into stiff fibrotic tissue through ECM remodeling. The stiff ECM forms a physical barrier for immune cell infiltration and favors an immunosuppressive landscape<sup>141</sup>. Upon reaching the tumor site, the CAR T cells have to extravasate and degrade the ECM to infiltrate the tumor. The enzyme heparanase (HPSE) is responsible for the degradation of heparin sulphate proteoglycans, one of the main ECM components. Unfortunately, during CAR T cells expansion HPSE expression is down regulated, limiting the solid tumor infiltration capacities of CAR T cells<sup>142</sup>.

Once the CAR T cells managed to infiltrate the tumor, they await a hostile immunosuppressive TME. The cells of the TME produce a range of cytokines which suppress TIL functionality while boosting tumor progression, such as TGF $\beta$ , IL-6, IL-10 and VEGF. High levels of these cytokines are found in the majority of ovarian tumors and malignant ascites, while they are absent or low in healthy ovarian tissues<sup>143 144</sup>. The elevated concentrations of IL-6 within ascites of ovarian cancer patients counts as an independent predictor of compromised progression-free survival, presumably due to the immunosuppression of T cells<sup>143 145</sup>. Tumor cells and TAMs produce TGF $\beta$ , which is a profound immune-suppressor by blocking HLA class II expression and deactivating TILs and NK cells. VEGF produced by CAFs, TAMs and tumor cells supports angiogenesis and suppresses immune responses by limiting dendritic cell maturation<sup>144 146</sup>. Ovarian cancer cells, Tregs and TAMs produce IL-10, which is a pleiotropic immune regulatory cytokine with potent immunosuppressive effects. Interestingly, IL-10 induces PD-1 expression on DCs allowing for immune evasion<sup>147</sup>. To improve the anti-tumor potency of CAR T cells in this hostile environment the balance in cytokines has to be shifted from anti-inflammatory to pro-inflammatory.

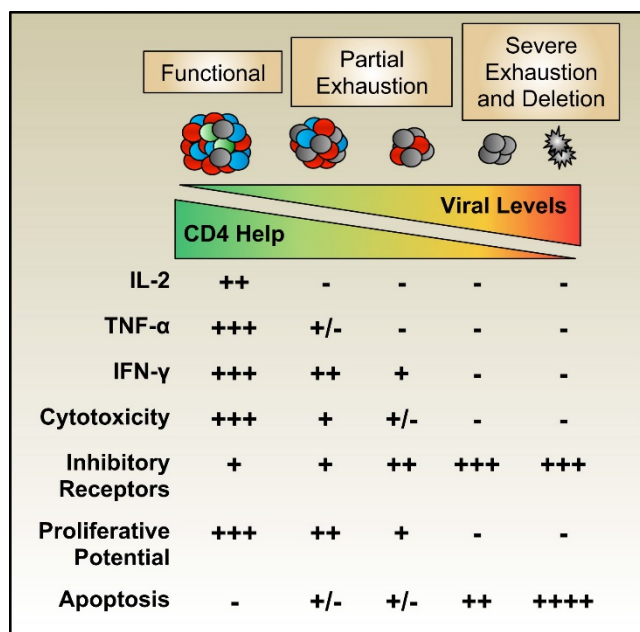
Disrupted cellular metabolism driven by hypoxia is another major hallmark of the TME. The uncontrolled tumor growth of solid tumors leads to hypoxia, and oxygen levels can range from

0,3% to 2,2% as compared to 1-14% under physiological conditions<sup>148</sup>. Hypoxia promotes the glucose uptake by tumor cells, promoting tumor growth and depleting T cells from glucose. The lack of nutrients such as glucose and essential amino acids (e.g. tryptophan) limits anti-tumor T cell effector functions<sup>149</sup>. Together with pro-inflammatory cytokines, hypoxia has been shown to promote the expression of the membrane-associated ecto-5'-nucleotidase CD73. In collaboration with CD39, the enzymatic activities of CD73 mediate a shift towards an immunosuppressive environment through the generation of adenosine. Accumulation of adenosine dampens T cell effector functions and stimulates anti-inflammatory responses through macrophages and Tregs amongst others<sup>150 151</sup>.

#### 1.7.4 Exhaustion

In the ideal setting, CAR T cells easily infiltrate the solid tumor and rapidly kill tumor cells. Upon antigen-recognition the CAR T cells are in killing mode, however, the solid tumor environment turns the odds in their favor through the previously described mechanisms<sup>87 152</sup>. Under these immunosuppressive circumstances it is extremely difficult for CAR T cells to efficiently kill all antigen positive tumor cells, which in turn leads to long-term antigen exposure. The repeated antigen exposure in combination with immunosuppressive factors progressively drive the T cells into a state of exhaustion. Exhaustion is a state of impaired T cell functionality, characterized by the gradual loss of proliferative capacity, cytolytic capacity, inflammatory cytokine production and altered transcriptional profile (**Figure 9**)<sup>153 154</sup>. The concept of exhaustion was first characterized in chronic viral infections and was subsequently validated in human cancer.

Chronic antigen stimulation and subsequent downstream TCR signaling is considered the major driving force behind T cell exhaustion. Extrinsic negative regulatory pathways (e.g. immunosuppressive cytokines) and cell-intrinsic negative regulatory signaling through co-inhibitory receptors (e.g. PD-1) further potentiate the progressive development of exhaustion<sup>153 155 156</sup>. T cell exhaustion is characterized by the hierarchical loss of T cell functions, including impaired IL-2, TNF and IFN $\gamma$  production, impaired cytolytic capacity and progressive phenotypic alterations through the co-expression of multiple co-inhibitory receptors. It is important to remember that co-inhibitory receptors are upregulated by activated T cells under physiological conditions to contract the immune response and therefore expression of one single CIM does not define a state of T cell exhaustion. However, the simultaneous expression of various co-inhibitory receptors is a phenotypic hallmark of exhaustion and CIMs can therefore also be referred to as exhaustion markers. The extent to which PD-1, TIM3, LAG-3 and CTLA-4 amongst others are co-expressed determines the severity of exhaustion. Different subgroups of exhausted T cells exist and T cells can recover from earlier stages of exhaustion (progenitor and partially exhausted T cells) while the later stage of terminally differentiated exhaustion is irreversible<sup>157-161</sup>. Tumor cells under immune attack upregulate the corresponding co-inhibitory ligands such as PD-L1 (PD-1 ligand) and HLA-DR (LAG-3 ligand), allowing for immune escape.



**Figure 9.** Hierarchical loss of T cell functions in response to chronic antigen exposure during viral infections, which is applicable in the cancer setting as well<sup>154 155</sup>.

### 1.7.5 Limited antigen availability and antigen escape

The success story of CD19-CAR T cell therapy in B cell malignancies can partly be attributed to the ideal expression pattern of CD19, being homogenously high on tumor cells at the start of treatment and selective expression by B cells. However, it has become apparent through long-term follow-up of CD19-CAR treated patients, that despite robust initial response rates of 70-90%, a substantial fraction of patients relapses and some of these relapses are CD19-negative or CD19-low<sup>162-164</sup>. Under CAR T cell mediated immune pressure, tumor cells modulate target-antigen expression to avoid immune control, a phenomenon referred to as antigen escape. In the setting of solid tumors, antigen escape poses an even greater obstacle to successful CAR T cell therapy due to the already existent antigen heterogeneity, as is the case for MSLN in ovarian cancer.

Tumor cells can employ several antigen escape mechanisms including lineage switch and antigen downregulation amongst others. In the case of lineage switch, disease recurs as a genetically related but phenotypically distinct cancer e.g. change from lymphoid to myeloid leukemia. Tumor cells can downregulate/lose cell surface expression of the target antigen, diminishing surface antigen levels below the threshold of CAR T cell activation. Another antigen escape mechanism described, entails the masking of the CD19 antigen on the surface of tumor cells by the CD19-CAR construct, which occurs when tumor cells were accidentally transduced during the CAR T cell production process<sup>162 165 166</sup>. Recently, CAR-mediated trogocytosis was identified as another culprit for antigen escape. Trogocytosis is the active process in which effector cells nibble off parts of the target cells, including the target-antigen, thereby decreasing antigen density on tumor cells. During trogocytosis, the target-antigen is not solely bitten off by effector cells, instead it is taken up, processed and cycled to the cell surface of effector cells for expression. Therefore, trogocytosis does not only impair CAR T cell functionality through limiting tumor antigen availability but also by promoting fratricide CAR T cell killing<sup>167 168</sup>.

Outside of the CAR T cell setting, trogocytosis is performed by several immune cells including macrophages, NK cells and conventional as well as non-conventional lymphocytes. Naïve and activated T cells are both capable of trogocytosis, although activated T cells are more efficient at extracting antigen from antigen-presenting cells<sup>169 170</sup>. Following trogocytosis, T cells serve as APCs and are capable of modulating the immune response. Overall the impact of trogocytosis on the immune response is dependent on the type of trogocytotic cell, as Tregs gain immunosuppressive capabilities through trogocytosis while trogocytotic CD4 helper T cells have the capacity to activate responding cells<sup>169 171</sup>. The immune modulatory impact of trogocytosis on trogocytotic T cells themselves is less well-described and remains to be elucidated. Recently, CD4 cells of the Th2 subset were shown to be more effective in trogocytosis than Th1 or non-polarized CD4 T cells and, regardless of starting subset, a Th2 phenotype was induced in CD4 T cells following trogocytosis<sup>172</sup>. In addition to promoting antigen escape and fratricide killing, trogocytosis by CAR T cells can impact the immune response due to changes in intracellular signaling, creating either an immunosuppressive or immunostimulatory environment.



## 2 RESEARCH AIMS

The main aim of the study was to evaluate the potential of MSLN-directed CAR T cell therapy for ovarian cancer. There are many bottlenecks to overcome for MSLN-CAR T cells in order to achieve successful clinical response rates in ovarian cancer, including antigen heterogeneity and the immunosuppressive tumor microenvironment. Introduction of a co-stimulatory domain has proven to be crucial for functional persistence of CAR T cells and in the challenging setting of advanced ovarian cancer, selection of the most advantageous co-stimulatory segment is of great importance. This brings us to the first two research aims of this PhD project: Evaluation of the impact of CD28- or 4-1BB co-stimulation on MSLN-CAR T cell functionality in several ovarian cancer models, both *in vitro* (**Paper I**) and *in vivo* (**Paper II**).

Developments in CAR T cell design, have resulted in a novel MSLN-CAR T cell construct with two distally mutated ITAM motifs in the CD3 $\zeta$  chain combined with CD28 co-stimulation, resulting in calibrated activation potential (M1xx). The last research aim addressed in **paper III** was to investigate the effect of tuned activation on functional MSLN-CAR T cell persistence in comparison to the two classical second-generation M28z and MBBz CAR T cell constructs.

### 3 MATERIALS AND METHODS

#### 3.1 HUMAN MATERIAL

Healthy donor buffy coats obtained through the Karolinska Universitetssjukhuset blood bank, Huddinge, were used as starting material for CAR T cell production in **Paper I-III**.

In **Paper II**, ascites of six patients suffering from metastatic HGSC were included.

#### 3.2 ETHICAL CONSIDERATIONS

The collection and use of material from HGSC patients in **Paper II** was approved by the Swedish Ethical Review Agency (Etikprövningsmyndigheten: 2016/1197-31/1, 2016/2060-32) and was performed by the Kaisa Lehti group (MTC, Karolinska Institute).

In **paper II** and **III**, animal experiments were performed with experimental laboratory mice. The usage of female NOD scid gamma (NSG) mice was approved by the Swedish Board of Agriculture (Jordbruksverket DNR 5.2.18-10712/2017, DNR 19354-2019) and all experiments were conducted according to the ethical permits and animal welfare law. NSG mice were purchased from Charles River laboratory and mice were housed in the Karolinska Universitetssjukhuset, Huddinge Animal facility according to EU regulations (individually ventilated cages, with up to 5 mice per cage, nest boxes and aspen-bedding). The mice were monitored daily by researchers and/or animal facility staff. The usage of mice was not taken lightly during these studies, all experiments were performed in compliance with regulations for laboratory animal welfare and everyone involved aimed to minimize distress caused to the NSG mice.

#### 3.3 LABORATORY METHODS

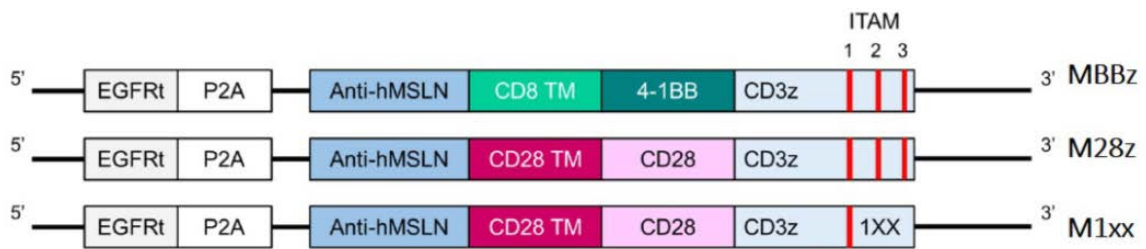
In this section, a concise overview of the *in vitro* and *in vivo* laboratory methods used in the making of **paper I-III** is presented. More details regarding performance of experiments and reagents used can be found in the enclosed papers.

##### 3.3.1 MSLN-CAR T cell transduction

At the core of **paper I-III**, is the production of MSLN-directed CAR T cells from healthy donor material. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats using Ficoll Paque density gradient centrifugation according to manufacturer's instructions. To support T cell proliferation and activation, PBMCs were cultured in activation medium consisting of AimV medium supplemented with 5% human serum (HS), 300 IU/mL Interleukin-2 (IL-2) and 50 ng/mL OKT-3 for two days. Following two days of activation, the T cells were harvested, counted and transferred to expansion medium (AimV + 5% HS + IL-2) for  $\gamma$ -retroviral transduction. RetroNectin coated 24-wells plates containing  $\gamma$ -retroviral supernatant were centrifuged at 2000g at 32 °C for 2 hours to bind the virus to the RetroNectin. Following centrifugation, the viral supernatant was removed from the wells and the T cells

were transferred to the RetroNectin and virus coated plates. The plates were spun down at 2000g at 32 °C for another 30 minutes, after which the plates were incubated overnight at 37°C, 5% CO<sub>2</sub>. The next days the T cells were harvested from the plates, counted and cultured in fresh expansion medium. CAR T cells were cultured for three to seven more days, during which the expansion medium was refreshed every other day and CAR transduction efficiency was assessed by flow cytometry as addressed in section 3.3.3.

The MSLN-directed CAR constructs were kindly gifted by Professor Michel Sadelain from MSKCC, NY, USA. All CAR constructs encode for an extracellular human scFv domain specific for MSLN (m912)<sup>123</sup> linked to a transmembrane domain followed by intracellular T cell derived signaling domains, and the truncated epidermal growth factor receptor (EGFRt) sequence via the 2A self-cleaving peptide (**Figure 10**). In **paper I** and **II**, two classical 2<sup>nd</sup> generation CAR T cells were studied, containing either a CD28 (M28z) or 4-1BB (MBBz) co-stimulatory domain linked to the CD3ζ domain. In **paper III**, a novel third MSLN-directed CAR construct was included, consisting of the CD28 co-stimulatory segment linked to a CD3ζ chain with point mutations in ITAM2 and ITAM3 (M1xx). A second generation CD19-CAR construct containing a CD28 co-stimulatory domain was provided by Professor S. Rosenberg and included as a control.



**Figure 10.** Structure of the three distinct MSLN-directed CAR T cells included in this thesis.

### 3.3.2 Human cancer cell lines

Several human cancer cell lines were used as target cells for MSLN-directed CAR T cells in **study I-III**. Three different ovarian cancer cell lines were included; SKOV-3 human ovarian tumor cells (Adenocarcinoma, ATCC, HTB-77) and the presumable HGSC cell lines OVCAR-3 (Adenocarcinoma, ATCC, HTB-161) and OVCAR-4 (gifted by Lehti group, Karolinska Institute) (both TP53 mutated). All three ovarian cancer cell lines express endogenous MSLN to a certain extent. K562 chronic myelogenous leukemia cells were included as MSLN-negative control cells.

To ensure stable and homogenous MSLN expression, all cell lines were transduced with human MSLN (variant 1) and/or green fluorescent protein (GFP)/firefly luciferase fusion protein (both constructs were SFG vectors and kindly donated by Prof. M. Sadelain, MSKCC). Transduced SKOV-3, OVCAR-3, OVCAR-4 and K562 tumor cells were sorted using fluorescence activated cell sorting (FACS) as explained later, to isolate MSLN<sup>high</sup>GFP<sup>+</sup> and MSLN<sup>low</sup>GFP<sup>+</sup> polyclonal pools.

### 3.3.3 Flow cytometry

Large part of the data presented in this thesis were generated by flow cytometry and its different applications. Flow cytometry is a versatile method, allowing for the detection of extracellular and intracellular characteristics of single cells. The principle of flow cytometry is based on labelling cells of interest with dyes and/or fluorochrome-conjugated antibodies targeting molecules inside the cell or on the cell surface and subsequently acquiring this cell suspension on a flow cytometer, which detects the light scattering and fluorescence characteristics at a single-cell level<sup>173</sup>. The labelled cell suspension is loaded into the fluidics system of the flow cytometer, which passes the cells one by one through the laser system where the cells are struck/excited by different lasers. Following laser excitation, the cells can emit light of different wavelengths which enter the optical system, where the signal is transmitted to detectors and subsequently amplified. The electronics system converts the amplified emitted light signals into digitized electronic signals, allowing for data display<sup>173</sup>. The flow cytometric data collected throughout **study I-III** were analyzed using FlowJo 10.7.2 software.

The field of flow cytometry has evolved in the last decades, starting as a single-parameter instrument to a multicolor machine, able to detect up to 40 different parameters<sup>174</sup>. In **paper I-III**, we have assessed up to 11 distinct parameters at a single-cell level. For reliable data generation using flow cytometry, several controls need to be included. In case of multicolor assays, compensation controls were included to correct for spectral overlap. Unstained control cells were incorporated to evaluate background fluorescence, and in some case, fluorescence minus one controls and/or isotype controls were included to allow for proper distinction between positive and negative populations. Furthermore, in the case of transduced cells (e.g. GFP+ tumor cells or CAR+ T cells), the proper biological controls in the form of untransduced cells were included in order to distinguish between transduced/untransduced fractions or as a negative control in stimulation assays.

#### 3.3.3.1 Phenotypic characterization

Phenotypic characterization of CAR T cells was performed in different settings throughout **paper I-III**, i.e. following transduction, after *in vitro* stimulation and for *ex vivo* analysis. Retroviral transduction of T cells with CAR constructs, results in CAR T cell products composed of transduced (CAR+) and non-transduced (CAR-) fractions. It is important to keep in mind that transduction efficiency and expression of CAR on the cell surface are not the same, since the CAR construct can be downregulated from the cell surface in transduced cells. Therefore, two different staining protocols were used to differentiate between these. The transduction efficiency was determined through EGFRt-directed stainings, while cell surface expression of the CAR construct was assessed by using an anti-scFv staining. Several antibody panels targeting extracellular molecules were included to evaluate the expression of different cell surface markers on CAR T cells as well as tumor cells.

### 3.3.3.2 FACS

Certain flow cytometers have the capacity to sort cells based on their fluorescent characteristics and this process is referred to as FACS (Fluorescence-Activated Cell sorting). The tumor cell lines used consistently throughout **paper I-III** were sorted using FACS to isolate MSLN<sup>high</sup>GFP+ cells as well as MSLN<sup>low</sup>GFP+ cells in **paper I**. Furthermore, in **paper I**, MSLN-transduced T cells were FACS sorted to isolate MSLN<sup>high</sup> and MSLN<sup>low</sup> populations. In **paper III**, *ex vivo* spleen-derived T cells were sorted using FACS to separate transduced (EGFRt+) T cells for gene expression analysis described later.

### 3.3.3.3 Intracellular cytokine staining

Flow cytometry can also be used to determine functionality of cells through intracellular cytokine stainings (ICS). MSLN-CAR T cells were co-cultured with MSLN<sup>high</sup> tumor cell lines in the presence of BrefeldinA and BD Golgi stop (contains Monensin), which block the secretion of the produced cytokines and thereby allowing for the intracellular detection of cytokines following permeabilization of the cells. Untransduced T cells and MSLN-negative target cells were included as background controls in ICS.

### 3.3.4 Magnetic-activated cell sorting

Another way to purify cell populations of interest is through magnetic-activated cell sorting (MACS). The process of MACS is based on labelling your cells of interest with magnetic beads linked to monoclonal antibodies and subsequently running this suspension through magnetic columns in a magnetic field. The labelled cells will bind to the magnetic column (positive fraction) and the unlabeled cells run through the column (negative fraction). The positive fraction is removed by separation of the column from the magnet and flushing of the column, this is referred to as positive selection. In **paper I**, CD4+ and CD8+ T cells were purified using positive selection with CD4 microbeads and CD8 microbeads, respectively. One advantage of MACS is the speed of the method, as samples can be sorted simultaneously in separate columns in the same magnetic field.

### 3.3.5 Lactate dehydrogenase cytotoxicity assays

During cell death, the plasma membrane ruptures resulting in the release of organelles and enzymes, including lactate dehydrogenase (LDH) into the environment. The LDH levels in cell medium can be quantified by a coupled enzymatic reaction, in which the concentration of LDH determines the amount of red formazan product measured by a spectrophotometer at 490 nm wavelength. In our setting, target cells were co-cultured with MSLN-CAR T cells or untransduced T cells for four and/or 24 hours after which the LDH concentration in the supernatant was quantified. Wells containing effector cells alone were included for background normalization. Supernatant of spontaneous release wells with target cells alone and maximum release wells containing lysed target cells were included to calculate target cell lysis in experimental wells.

### 3.3.6 Chromium-51 release assays

The radioactive isotope of the metallic element chromium, chromium-51 ( $\text{Cr}^{51}$ ) has been widely used for the quantification of cytotoxicity. Target cells are labelled with  $\text{Cr}^{51}$  prior to co-culture with effector cells. In **paper I**,  $\text{Cr}^{51}$  release assays were performed to assess bystander killing of MSLN-negative target cells. MSLN-CAR T cells or untransduced T cells were co-cultured with 1)  $\text{Cr}^{51}$ -labelled MSLN-negative K562 cells alone ( $\text{ctrl}^{\text{Cr}^{51+}}$ ) or 2) in combination with 50% MSLN-positive  $\text{Cr}^{51}$ -unlabelled K562 cells ( $\text{MSLN}^{\text{Cr}^{51-}}$ ). Supernatant was collected following four hours of incubation and transferred to a 1450 MicroBeta Liquid Scintillation counter (Perkin Elmer) for  $\text{Cr}^{51}$  release measurement. Mechanically lysed target cells were used as a reference for maximum  $\text{Cr}^{51}$  release and the supernatant from wells containing target cells only was a measure for spontaneous lysis. Bystander killing was identified as increased lysis of  $\text{ctrl}^{\text{Cr}^{51+}}$  cells in the presence of unlabeled MSLN-positive cells as compared to lysis of  $\text{ctrl}^{\text{Cr}^{51+}}$  cells alone.

### 3.3.7 Incucyte® live-cell analysis

In order to assess killing of target cells in a 3D *in vitro* settings, tumor spheroids were generated using SKOV-3 cells with different MSLN frequencies. The tumor spheroids were co-cultured with M28z, MBBz or untransduced T cells in the Incucyte® S3 Live-Cell imaging system, allowing for quantification of real-time killing during 24 hours. Caspase is activated during cell death and addition of CellEvent™ caspase 3/7 green detection reagent to cultures allows for the quantification of apoptosis. Activated caspase in apoptotic cells can cleave the CellEvent™ caspase 3/7 green detection reagent, generating fluorescent emission which can be detected by the green fluorescent channel of the Incucyte S3 system.

### 3.3.8 FluoroSpot

Over 50 years ago, enzyme-linked immunosorbent assays (ELISA) were developed allowing for robust quantification of a single analyte in suspension e.g. blood. The technique is plate-based, coating plates with antibodies or antigens specific for the soluble analyte of interest. Binding of the analyte to the coated plates can be detected through an enzymatic reaction and subsequent absorbance read-out of the plate using a spectrophotometer<sup>175 176</sup>. Since the introduction of ELISA in the 1970s, advancements have been made in enzyme-linked immunoassays, resulting in development of enzyme-linked immunospot (ELISpot) and the adaptation to FluoroSpot assays. The major advantage of FluoroSpot assay is the capacity to detect various analytes simultaneously at a single-cell level. Instead of detecting analytes in biological fluids, cells are directly incubated together with their respective stimulus on plates coated with different capture antibodies. The plates are washed following incubation and the plate-bound analytes are detected by using fluorescence-conjugated reagents allowing for quantification of the fluorescence signal on the FluoroSpot reader (IRIS, Mabtech).

### 3.3.9 Preclinical *in vivo* models

In **paper II** and **III**, mice models were incorporated to study the efficacy of MSLN-directed CAR T cells. Female NSG mice were inoculated with MSLN<sup>high</sup>GFP/luc<sup>+</sup> SKOV-3 or OVCAR-4 tumor cells through microsurgery in the left ovary or intraperitoneal (i.p.) injection, respectively. In **paper II**, mice were treated with MSLN-CAR T cells or control T cells through intravenous (i.v.) injection, whereas in **paper III**, mice received CAR T cells i.v. in the SKOV-3 model and i.p. in the OVCAR-4 model. To longitudinally characterize CAR T cells and tumor cells *in vivo*, intermediate sacrificing time points were included in the SKOV-3 model in **paper II** and **III** and in the OVCAR-4 model in **paper III**. The remaining mice were kept for survival analysis.

### 3.3.10 Processing of mice tissues

For characterization of MSLN-CAR T cells and tumor cells *ex vivo*, blood, primary SKOV-3 tumors/ovarian tissue, organs (spleen and lungs) and ascites (when present) were collected upon sacrifice of mice. The majority of primary tumor/ovarian tissue, lungs and spleens were processed into a single-cell suspension and subsequently filtered through a 100  $\mu$ M strainer to remove clumps. The spleen, ascites and blood were treated with a red blood cell lysis buffer. The organ-derived single-cell suspensions were stained with antibodies for flow cytometric analysis. In case of sufficient primary tumor/ovarian tissue and/or spleen material, parts of the tissues were cut into small pieces and cultured in T cell medium (Aim V + 5% human serum + IL-2) for 1 to 4 days, prior to use in *ex vivo* functional CAR T cell assays. In the OVCAR-4 model in **paper III**, the spleen-derived single-cell suspension was used for phenotypic characterization of CAR T cells as well as FACS sorting of CAR T cells. In **paper II**, a fraction of the primary SKOV-3 tumors/ovarian tissues were fixed in paraformaldehyde and stored in 70% ethanol until further processing for immunohistochemistry analysis.

### 3.3.11 Immunohistochemistry and Immunofluorescence

Similar to flow cytometry, antibodies were used to visualize antigens in immunohistochemistry (IHC) and immunofluorescence (IF). However, instead of antigen detection on a single-cell level, IHC and IF allow for the detection of cells and their antigens location within a tissue section. The detection and visualization of antigens in IHC depends on chromogenic reactions and brightfield microscopy, whereas in IF, fluorochrome-conjugated antibodies and confocal/fluorescence microscopy are used. In **paper I**, IF was used to visualize T cell infiltration as well as MSLN distribution in paraformaldehyde-fixed SKOV-3 tumor spheroids. In **paper II**, tumor microarray (TMAs) sections were created from paraformaldehyde-fixed tumor material of sacrificed mice and used for visualization of T cell infiltration and MSLN distribution through IHC. Quantification of IHC and IF data was performed using QuPath.

### 3.3.12 Luminex

In **paper II**, we performed magnetic human multiplex assays on plasma samples collected upon sacrifice of SKOV-3 inoculated mice and supernatant of *ex vivo* stimulated CAR T cells. The

luminex multiplex assay principle is comparable to ELISAs, although way more advanced allowing for the detection of many analytes at once. Two different cocktails with magnetic beads conjugated to different analyte-specific antibodies were used, allowing for the detection of up to 13 analytes simultaneously. Binding of respective analytes is detected via fluorescently-conjugated reagents and the fluorescent signal was quantified with the Luminex MAGPIX system, which can characterize each specific bead through its unique fluorescence signal<sup>177</sup>.

### **3.3.13 Gene expression analysis**

In **paper III**, M28z and M1xx CAR T cells were isolated from mice spleen upon sacrifice through FACS. Purified MSLN-CAR T cells were pelleted and lysed upon gene expression analysis using the NanoString nCounter CAR-T Characterization and the nCounter Metabolic Pathways Panels. The nCounter analysis system from NanoString allows for quantification of the transcriptome through detection of hundreds of different individual RNA molecules simultaneously, through usage of unique fluorescent barcodes allowing for digital detection of specific target molecules. This method is highly sensitive and can be performed using a minimal amount of cells. The data were analyzed in collaboration with an external expert using R. Lowly expressed genes were excluded, with a cutoff based on the maximum signal among the negative controls. Batch variations were removed by subtracting the average overall difference between the replicates and remaining differences were considered actual dispersion, which was estimated using the Bayesian approach of DEseq2.

## **3.4 STATISTICAL ANALYSIS**

Data analysis, graphing and statistical analysis were accomplished by using GraphPad Prism software version 8. Wilcoxon matched pairs signed rank tests and Friedman tests followed by Dunn's multiple comparisons tests were used to compare two or more groups of paired samples, respectively. Unpaired samples were analyzed using Mann-Whitney (two groups) or Kruskal-Wallis ( $\geq$  three groups) tests. For comparison between groups of samples over time as was the case in Incucyte and mice experiments, one-way and two-way ANOVA tests were used, respectively. For generation of survival curves, the Kaplan-Meier method was performed and statistical differences in survival between groups was assessed by the log-rank test. To determine correlation between two factors, linear regression and/or Spearman correlation tests were performed. In order to assess similarity and dissimilarity between groups of samples, based on  $\geq 2$  factors, unbiased hierarchical clustering was performed using the CIMminer software. Clustering results were visualized in two-dimensional clustering image maps and dendograms (Euclidean distance).

## 4 RESULTS AND DISCUSSION

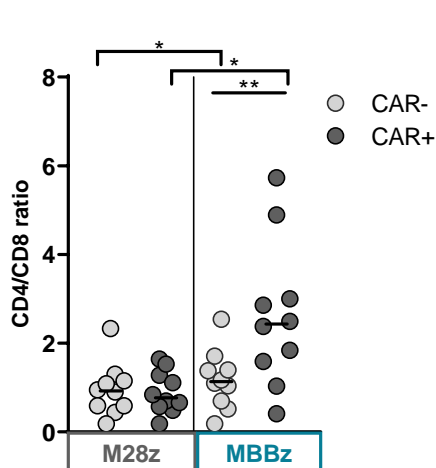
The unprecedented clinical results achieved with 2<sup>nd</sup> generation CD19-CAR T cell therapy caused a paradigm shift within the field of immunotherapy. The possibility to directly target surface antigen in a HLA-independent manner combined with full-on activation of T cell effector functions was groundbreaking and opened doors for future immunotherapies. Since the success of CAR T cell therapy in hematological malignancies, the quest for successful translation to solid tumors started. However, this journey has not been without setbacks and to date, clinical response rates remain marginal in solid tumors including ovarian cancer.

The overall aim of this thesis is to validate the potential of MSLN-directed CAR T cells for the treatment of ovarian cancer, with a specific focus on the impact of co-stimulation on MSLN-CAR T cell functionality (**paper I-II**) and the calibration of CAR T cell activation (**paper III**). Throughout all papers, we aim to elucidate the mechanisms behind MSLN-CAR T cell functionality. For this a combination of *in vitro* (**paper I**) and preclinical *in vivo* models (**paper II-III**) were explored.

### 4.1 FUNCTIONALITY OF CD4+ AND CD8+ M28Z AND MBBZ CAR T CELLS

During CAR T cell transduction, some T cells will be transduced with the MSLN-CAR construct, whereas the other fraction remains non-transduced. Transduction efficiency was determined by EGFRt staining and transduced T cells are referred to as either CAR+ or EGFRt+ and non-transduced as CAR- or EGFRt- in all papers. Of note, anti-scFv staining was used to identify cell surface expression of the CAR construct in certain figures and these cells are also referred to as CAR+ as well, which was then specifically indicated in the corresponding figure legends.

Eight days after MSLN-CAR transduction, the CD4/CD8 ratio was different between M28z- and MBBz-transduced T cells. The CD8+ T cell population was more frequent in M28z- than

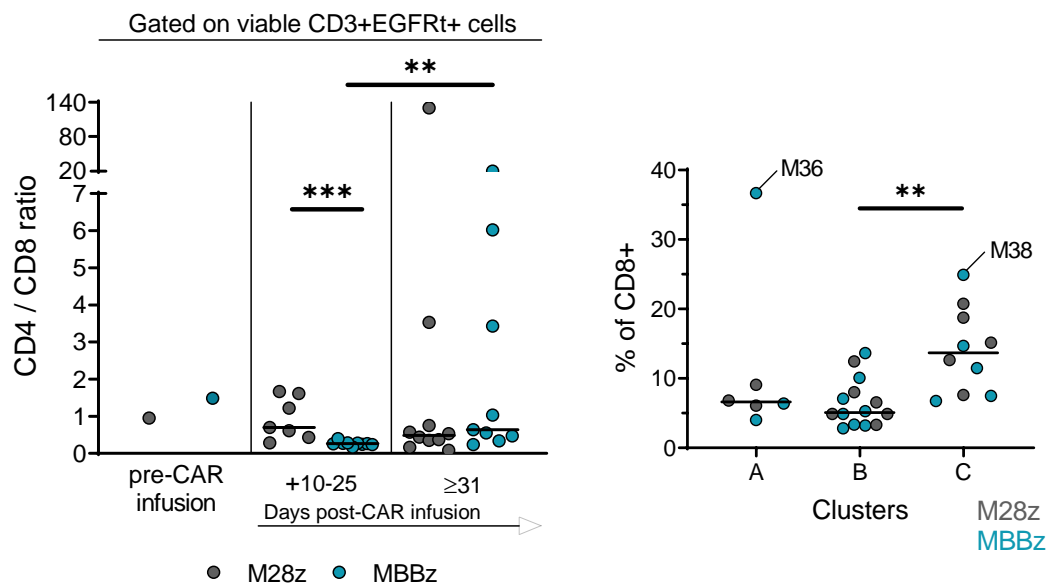


MBBz-transduced T cells, while the CD4+ population was predominant in MBBz CAR T cells. The skewness of MBBz-transduced T cells towards the CD4+ population was specific for the CAR+ fraction, while the CD4/CD8 ratio was comparable within the CAR+ and CAR- fractions of M28z-transduced T cells (**Figure 11**).

**Figure 11.** CD4/CD8 ratio within transduced (CAR+) and non-transduced (CAR-) fractions following M28z or MBBz CAR T cell transduction. Derived from **paper I**.

To investigate whether the bias towards the CD4<sup>+</sup> population in MBBz CAR T cells could affect killing capacity, the individual cytotoxic potential of CD4<sup>+</sup> and CD8<sup>+</sup> M28z- and MBBz-transduced T cells was evaluated. No differences in lysis of MSLN<sup>high</sup> SKOV-3 cells were detected between CD4<sup>+</sup> enriched, CD8<sup>+</sup> enriched and CD4/CD8 mixed MSLN-CAR T cells. However, CD4<sup>+</sup> enriched M28z and MBBz CAR T cells were inferior in killing MSLN<sup>high</sup> OVCAR-3 cells compared to CD8<sup>+</sup> enriched and CD4/CD8 mixed MSLN-CAR T cells. The molecular signature of OVCAR-3 cells resembles the highly aggressive form of ovarian cancer, HGSC, more than that of SKOV-3 cells, which are unlikely to be HGSC. Harboring the characteristic *TP53* mutation, OVCAR-3 cells are capable of uninhibited proliferation and evading apoptosis<sup>178 179</sup>. These data suggest that in a challenging environment, as is the case in HGSC, CD4<sup>+</sup> M28z and MBBz CAR T cells require support from CD8<sup>+</sup> T cells to achieve tumor clearance.

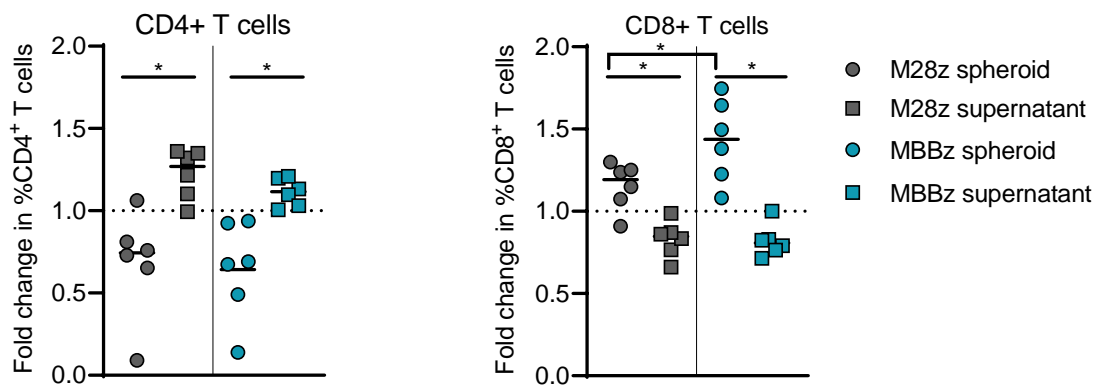
Interestingly, we observed a shift in the CD4/CD8 ratio of MBBz CAR T cells *in vivo* as compared to the starting product in **paper II**. The CD8<sup>+</sup> population was predominant in tumor-infiltrating MBBz CAR T cells, especially 10-25 days after CAR infusion. Increased incidence of intratumoral CD8<sup>+</sup> MSLN-CAR T cells was linked to enhanced survival in **paper II**, and levels of CD8<sup>+</sup> TILs were significantly higher in mice with a survival advantage (cluster C) (**Figure 12**).



**Figure 12.** On the left, ratio of CD4/CD8 in M28z and MBBz CAR T cells prior to infusion and upon *ex vivo* analysis. Frequency of intratumoral CD8 T cells in cluster C mice (prolonged survival) and cluster B mice (poor survival) on the right. Derived from **paper II**.

The data in **paper II** do not clarify whether the increased CD8<sup>+</sup> frequency in intratumoral MBBz CAR T cells is a result of superior tumor infiltration by CD8<sup>+</sup> as compared to CD4<sup>+</sup> T cells and/or due to outgrowth of CD8<sup>+</sup> over CD4<sup>+</sup> TILs. In **paper I** it was shown that CD8<sup>+</sup> M28z and MBBz CAR T cells had superior spheroid infiltration capabilities as compared to their CD4<sup>+</sup> counterparts and instead of infiltrating the MSLN<sup>high</sup> SKOV-3 tumor spheroids,

CD4<sup>+</sup> T cells remained to a higher frequency within the supernatant (**Figure 13**). The fold change in frequency of CD8<sup>+</sup> T cells from starting product to spheroid-infiltrated T cells was significantly higher in MBBz than M28z CAR T cells, implying CD8<sup>+</sup> MBBz CAR T cells were more efficient in spheroid infiltration. In **paper III** we observed a shift in the CD4/CD8 ratio in MBBz CAR T cells towards the CD8<sup>+</sup> population following two *in vitro* stimulations. This means there was an outgrowth of CD8<sup>+</sup> CAR T cells and/or a decline in the population of CD4<sup>+</sup> T cells following target antigen exposure.



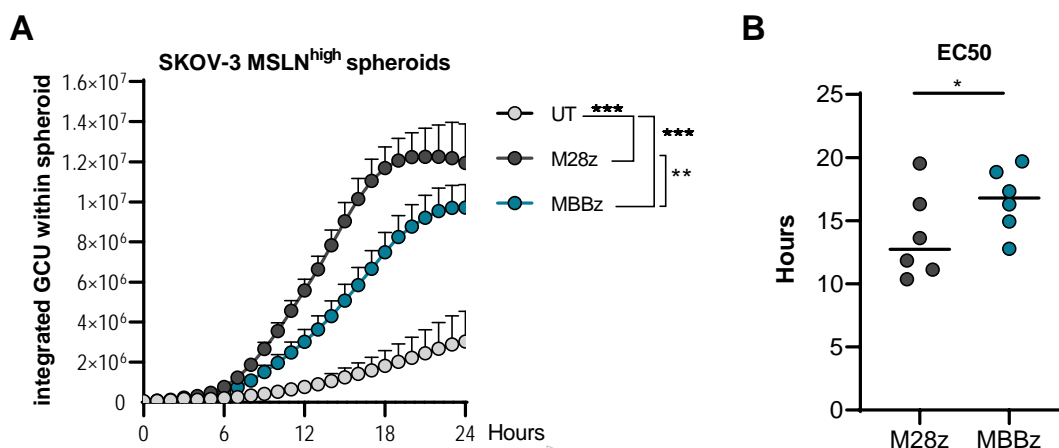
**Figure 13.** Fold change in CD4 and CD8 frequency of M28z and MBBz CAR T cells following culture with MSLN<sup>high</sup> SKOV-3 tumor spheroids relative to starting point. Derived from **paper I**.

Together, these data suggest that the skewness towards the CD4<sup>+</sup> phenotype following MBBz CAR transduction does not negatively impact cytotoxic potential *in vivo* relative to M28z CAR T cells, as CD8<sup>+</sup> MBBz CAR T cells are effective in tumor infiltration and possibly, proliferation following antigen stimulation. As previously mentioned several CD4 helper subsets have been identified, each with their own specific effector functions and certain subsets are considered anti-tumorigenic (Th1) while others elicit pro-tumorigenic effects (Tregs, Th2)<sup>36,37</sup>. Therefore, it would be of interest to characterize the CD4 helper subset composition following MSLN-CAR transduction as well as repeated antigenic stimulation, to determine whether there is a shift in the CD4 subset population.

## 4.2 M28Z CAR T CELLS DISPLAYED ENHANCED CYTOLYTIC CAPACITY COMPARED TO MBBZ CAR T CELLS

In order to assess cytolytic capacity of MSLN-CAR T cell in an *in vitro* 3D model, tumor spheroids were generated using MSLN<sup>high</sup> SKOV-3 cells of different MSLN frequencies (MSLN<sup>high</sup>, MSLN<sup>high/low</sup> and MSLN<sup>low</sup>). Lysis of tumor spheroids by M28z and MBBz CAR T cells was monitored over 24 hours using the IncuCyte live-cell analysis system. Regardless of MSLN expression levels on SKOV-3 spheroids, M28z CAR T cells elicited superior tumor lysis relative to MBBz CAR T cells (**Figure 14A**). MBBz CAR T cells were capable of targeting MSLN<sup>high</sup> and MSLN<sup>high/low</sup> spheroids but not MSLN<sup>low</sup> tumor spheroids, while M28z CAR T cells did effectively target MSLN<sup>low</sup> spheroids. Interestingly, M28z and MBBz CAR T cells displayed distinct killing kinetics. M28z CAR T cells lysed MSLN<sup>high</sup> SKOV-3 spheroids

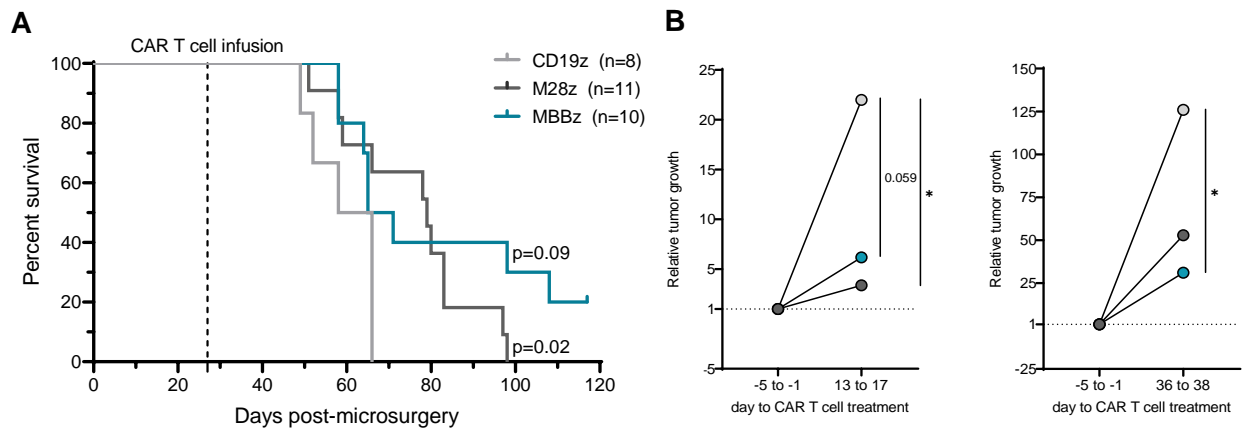
more rapidly than MBBz CAR T cells as indicated by the shorter time to EC50 (12.74 versus 16.80 hours, respectively) (**Figure 14B**).



**Figure 14. A)** Real-time killing of MSLN<sup>high</sup> SKOV-3 spheroids by untransduced T cells (UT), M28z or MBBz CAR T cells. **B)** EC50 values reveal time point on which 50% of maximum tumor spheroid killing was achieved. Derived from **paper I**.

Similar findings were made in a preclinical orthotopic model of ovarian cancer using MSLN<sup>high</sup> SKOV-3 tumor cells in **paper II**. Treatment with M28z CAR T cells significantly prolonged survival compared to control mice (CD19-CAR). Response rates following MBBz CAR T cell infusion were lower, and hence, no significant survival advantage was detected (**Figure 15A**). Importantly, in responders, MBBz CAR T cells elicited tumor regression and some mice were considered to be under long-term remission (2 out of 10). M28z CAR T cell treatment did not result in remission and the treatment effect was transient, merely delaying tumor progression. These findings were reflected in the relative tumor growth, displaying an initial rapid treatment effect elicited by M28z CAR T cells and this advantage was lost at later time points, when MBBz CAR T cell treatment significantly impeded tumor progression (**Figure 15B**).

The *in vitro* and *in vivo* results described reinforce one another, demonstrating distinct killing kinetics between CD28 and 4-1BB co-stimulated MSLN-CAR T cells. M28z CAR T cells rapidly eliciting potent but short-term anti-tumor responses, while 4-1BB co-stimulation enhanced functional persistence of CAR T cells. Due to these different cytolytic kinetics, it would be of interest to investigate the therapeutic effect of simultaneous and consecutive treatment with M28z and MBBz CAR T cells. For instance, what would the impact be of targeting the large tumor bulk with M28z CAR T cells followed by consecutive treatment with MBBz CAR T cells or dual infusion of M28z and MBBz CAR T cells? Combination of M28z and MBBz CAR T cell therapy can potentially induce potent and fast anti-tumor responses combined with long-term tumor control.



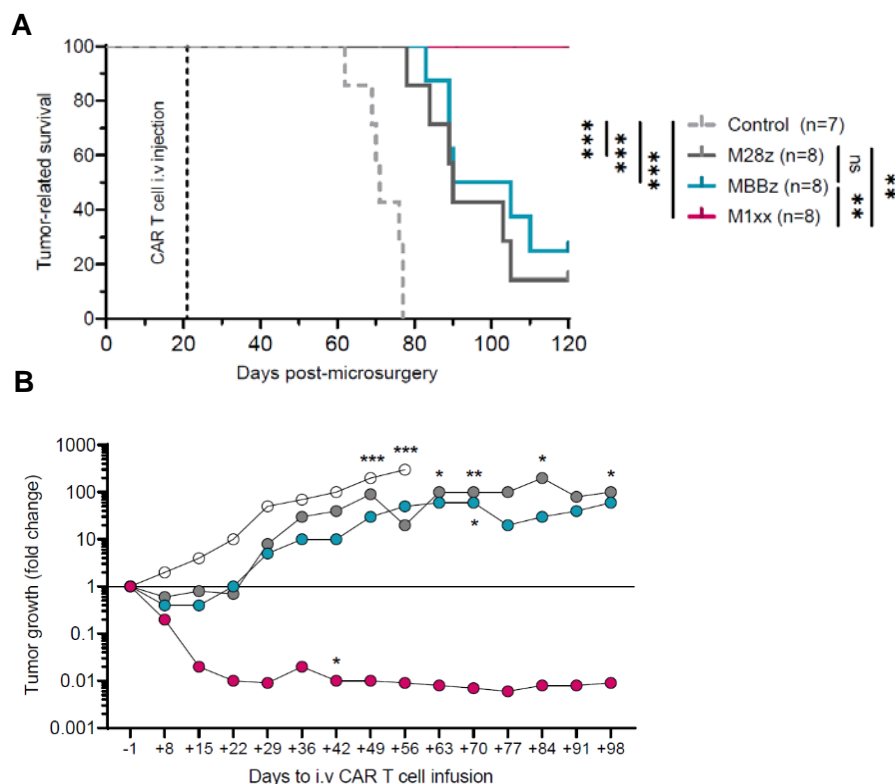
**Figure 15.** A) Survival curves of SKOV-3 inoculated mice following treatment with CAR T cells. B) Relative tumor growth in response to CAR T cell treatment as compared to tumor burden prior to treatment. Derived from paper II.

#### 4.3 CALIBRATION OF CAR ACTIVATION RESULTS IN SUPERIOR CYTOTOXIC CAPACITY *IN VIVO*

In **paper III**, the classical second generation CAR constructs, M28z and MBBz, were evaluated in comparison to the novel M1xx CAR construct, which contains only one functional ITAM domain in the CD3 $\zeta$  chain and a CD28 co-stimulatory domain.

No obvious differences were detected in functionality as determined by *in vitro* cytokine production between M28z, MBBz and M1xx CAR T cells following repeated antigen-stimulation. More importantly, M1xx CAR T cell treatment mediated remarkable anti-tumor responses in the orthotopic MSLN<sup>high</sup> SKOV-3 model in **paper III**. Treatment with M1xx CAR T cells was extremely effective, as substantial tumor regression was observed in 100% of the M1xx-treated mice (**Figure 16A-B**). In fact, M1xx-mediated tumor control was so successful that >90 days post treatment, tumor burden was still on the threshold of detection, indicating M1xx-treated mice were under long-term remission. Despite all three MSLN-CAR T cell products significantly augmenting survival as compared to control mice, M28z and MBBz CAR T cells were not able to induce persistent tumor regression and solely M1xx-treated mice achieved long-term remission.

To mimic disseminated HGSC, mice were inoculated with MSLN<sup>high</sup> OVCAR-4 tumor cells through i.p. injections in **paper III**. Mice received locally administered (i.p.) M28z, M1xx or control T cells. In this challenging setting of peritoneal carcinomatosis, treatment with M1xx CAR T cells did not result in persistent tumor regression as observed in the orthotopic SKOV-3 model. Tumor progression was significantly delayed in response to M28z and M1xx CAR T cell treatment as compared to control mice, however, M1xx CAR T cell treatment augmented survival more than M28z CAR T cell treatment. M1xx CAR T cells elicited rapid and potent anti-tumor responses after CAR infusion, with a dramatic decline in tumor growth shortly after treatment (+7 days). Despite the rapid initial treatment effect, the tumor progressed slowly but steadily and tumor burden reached initial levels again 56 days post M1xx CAR T cell treatment, as compared to 35 days in M28z-treated mice (**Figure 16B**).



**Figure 16.** A) Survival curves of SKOV-3 inoculated mice following treatment with CAR T cells. B) Tumor growth following CAR T cell treatment as determined by fold change in tumor burden (day -1). Derived from **paper III**.

Together, these data show that calibration of MSLN-CAR T cell activation provides superior tumor control in preclinical models of ovarian cancer as compared to classical second generation MSLN-CAR constructs. However, in the setting of disseminated HGSC, there are limitations to the superiority of M1xx CAR T cells, as all mice succumb to disease progression despite a powerful initial anti-tumor response. The solid tumor niche has proven to be an extremely hostile environment for anti-tumorigenic immune cells, including CAR T cells. Throughout all papers included in this thesis, we aimed to elucidate the immune escape mechanisms affecting functional persistence of M28z, MBBz and M1xx CAR T cells in various models of ovarian cancer as discussed further below.

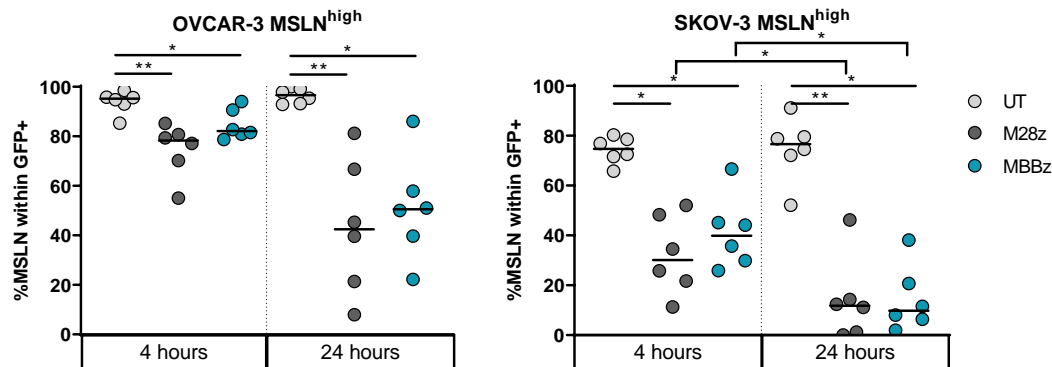
## 4.4 IMMUNE ESCAPE MECHANISMS

### 4.4.1 Loss of MSLN surface expression by tumor cells

To ensure stable and high MSLN expression throughout experiments, all cell lines were transduced with MSLN (regardless of parental MSLN expression in ovarian cancer cell lines) and subsequently sorted to isolate MSLN<sup>high</sup> target cells. However, one phenomenon we consistently detected throughout **paper I-III**, is the loss of MSLN surface expression by MSLN<sup>high</sup> OVCAR-3 and SKOV-3 cells.

In **paper I**, exposure of MSLN<sup>high</sup> OVCAR-3 and SKOV-3 cells to M28z or MBBz CAR T cells, resulted in a dramatic reduction of MSLN expression by the respective target cells

(**Figure 17**). The decline in MSLN surface expression by target cells was already significant following four hours of incubation with MSLN-CAR T cells and continued to gradually decrease over 40% during 24 hours of co-culture. The loss in MSLN expression was CAR-mediated as the frequency of MSLN<sup>+</sup> tumor cells remained stable during co-culture with untransduced control cells.



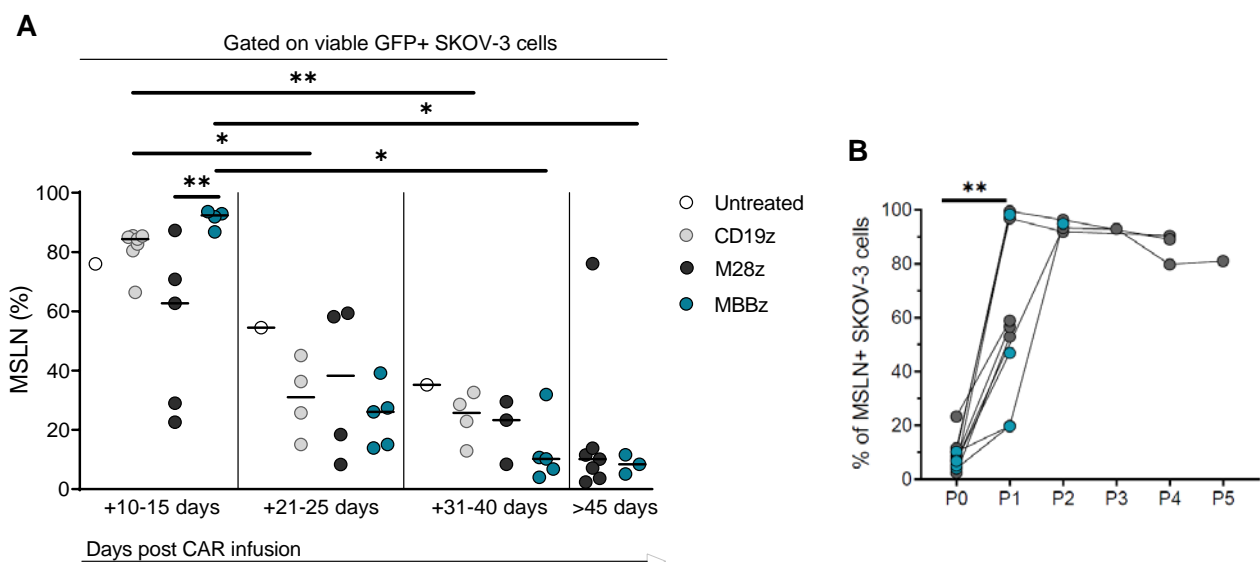
**Figure 17.** MSLN surface expression on OVCAR-3 and SKOV-3 target cells during co-culture with UT, M28z or MBBz CAR T cells. Derived from **paper I**.

The progressive loss of MSLN surface expression by MSLN-transduced MSLN<sup>high</sup> target cells was not merely an *in vitro* phenomenon, as similar observations were made in the orthotopic preclinical SKOV-3 tumor model in **paper II** and **III**. Due to inclusion of intermediate sacrificing time points and natural humane endpoints in **paper II**, we were able to quantify MSLN surface expression by primary SKOV-3 tumor cells over time. Prior to tumor inoculation through microsurgery, MSLN expression ranged between 95-97% and was still >80% upon CAR T cell treatment. We detected a gradual and drastic reduction in MSLN surface expression by primary SKOV-3 tumor cells in all treatment groups (MSLN-CAR or CD19-CAR T cell treatment). Interestingly, following M28z CAR T cell treatment, the frequency of MSLN<sup>+</sup> SKOV-3 tumor cells decreased more steeply as compared to MBBz-CAR T cell treatment (**Figure 18A**). In **paper III**, once again MSLN surface expression decreased drastically on primary SKOV-3 tumor cells, with median levels decreasing from 94% prior to tumor inoculation to below 20% at time of sacrifice regardless of treatment modality. One striking difference between the *in vitro* results in **paper I** and *in vivo* results of **paper II-III**, is the specific CAR-mediated loss of MSLN surface expression by tumor cells *in vitro*, while there was an overall reduction in MSLN expression levels on primary SKOV-3 cells *in vivo* regardless of treatment (control or MSLN-CAR T cells).

Discrepancies between *in vitro* and *in vivo* results are not uncommon, since the *in vivo* environment is far more complex with many more confounding factors than in an *in vitro* experimental setting. In saying that, several mechanisms are at play in our preclinical models in modulating MSLN surface expression by tumor cells. Our *in vivo* data imply there was CAR-mediated loss of MSLN expression in parallel with other mechanisms at play. The rapid decrease in frequency of MSLN<sup>+</sup> SKOV-3 cells in response to M28z CAR T cell treatment as compared to MBBz CAR T cells in **paper II**, is indicative of rapid anti-tumor pressure exerted by the M28z CAR T cells. In concordance with this, MSLN expression levels on primary

SKOV-3 tumor cells negatively correlated with tumor weight upon sacrifice in the control treatment group and not in MSLN-CAR T cell treatment groups (**Paper III**), implying that in the absence of antigen-targeted therapy, MSLN surface expression gradually decreased as the primary tumor progressed while MSLN-CAR T cell treatment mediated an accelerated loss of surface antigen expression.

Importantly, MSLN surface expression by SKOV-3 cells was not homogenously lost as high frequencies of MSLN+ tumor cells were detected in lung and ascitic metastatic sites in **paper II**. Furthermore, both in **paper II** and **III**, metastatic MSLN+ tumor cells were detected in the spleen of sacrificed mice. This could be explained by tissue-specific regulation of MSLN surface expression or be a reflection of the time of metastasis, in which early onset of metastasis, when the frequency of MSLN+ tumor cells was still high within the primary tumor, would probably result in MSLN+ tumor metastasis. However, the overall reduction in MSLN surface expression regardless of treatment modality as well as the extracellular re-expression of MSLN by SKOV-3 cells *ex vivo* in **paper II** (**Figure 18B**), imply that the loss of MSLN surface expression in the primary tumor is not the result of selection against MSLN+ tumor cells. Despite the lack of MSLN detection on the cell surface of tumor cells by flow cytometry, IHC revealed that the majority of primary tumor cells were MSLN+, suggesting down-regulation of MSLN surface expression and intracellular accumulation of the MSLN protein. Taken together, our data imply that expression of MSLN by tumor cells is actively regulated through antigen internalization and cell surface recycling, under control of environmental/organ-specific cues as well as MSLN-CAR T cell mediated pressure. It would be of great interest to further elucidate the MSLN expression kinetics. For this additional investigations regarding the cellular localization of MSLN in different environmental settings are key.



**Figure 18.** A) MSLN surface expression upon *ex vivo* analysis of SKOV-3 tumor cells and B) during *ex vivo* culture for several passages (P). Derived from **paper II**.

#### 4.4.2 Upregulation of CIMS by tumor cells *in vivo*

Tumor cells are flexible and ever-adapting to cues from the environment and the remaining MSLN<sup>+</sup> tumor cells had to find alternative ways to escape immune control. One mechanism through which tumor cells can evade killing by T cells is through the upregulation of CIMs. In line with this, in **paper II-III**, MSLN<sup>+</sup> SKOV-3 tumor cells were shown to specifically upregulate expression of co-inhibitory ligands *in vivo*. PD-L1 and HLA-DR expression gradually increased over time and, in particular, by the MSLN<sup>+</sup> SKOV-3 tumor cells as compared to their MSLN<sup>-</sup> counterparts.

This immune escape mechanism is employed under substantial immune pressure and hence, expression of co-inhibitory ligands is indicative of immune infiltration. Presence of PD-L1<sup>+</sup> and/or HLA-DR<sup>+</sup> tumor cells has been associated with favorable prognosis in certain cancers, including ovarian cancer<sup>180 181</sup>. In line with this, expression of HLA-DR alone or in combination with PD-L1 and/or Galectin-9 was elevated in mice with prolonged survival (Cluster C, **paper II**). This cluster of mice was characterized by increased levels of CD8<sup>+</sup> TILs and high plasma concentrations of IFN- $\gamma$  and TNF amongst others, indicative of a strong immune response. Furthermore, in **paper III** we detected specific upregulation of the death receptor FAS on MSLN<sup>+</sup> tumor cells. Together our data imply that the remaining MSLN<sup>+</sup> tumor cells were under substantial immune pressure and for their survival, they required increased upregulation of CIMs as compared to their MSLN<sup>-</sup> counterparts.

#### 4.4.3 Exhaustion of MSLN-CAR T cells

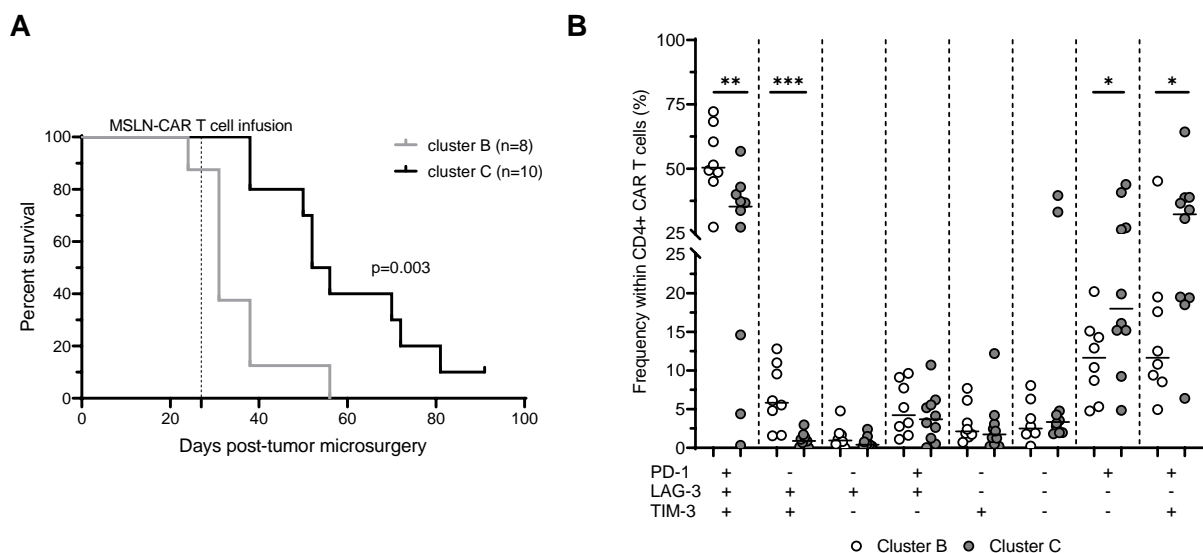
It is important to keep in mind that despite the terminology of exhaustion marker, the expression of one particular CIM does not necessarily imply T cell exhaustion and instead might represent T cell activation. However, the simultaneous expression of several CIMs is indicative of T cell exhaustion<sup>154 159</sup>.

##### 4.4.3.1 CD28 and 4-1BB co-stimulation impact MSLN-CAR T cell exhaustion differently

The frequency of PD-1/LAG-3/TIM-3 triple positive T cells, indicative of phenotypic exhaustion, was significantly higher following transduction with the M28z than MBBz CAR construct as described in **paper I**. PD-1 and LAG-3 expression levels were upregulated by both M28z and MBBz CAR T cells in response to *in vitro* antigen stimulation. Despite this upregulation, the frequency of PD-1/LAG-3/TIM-3 triple positive cells remained higher in M28z as compared to MBBz CAR T cells during initial (**paper I**) and repeated (**paper III**) *in vitro* antigen exposure. Despite the earlier and stronger signs of exhaustion, M28z CAR T cells displayed superior cytolytic capacity against SKOV-3 tumor spheroids relative to MBBz CAR T cells in **paper I**, suggesting M28z CAR T cells were not functionally exhausted *in vitro* and were capable of rapidly eliciting effector functions. Of note, the degree of exhaustion marker expression was dependent on *in vitro* culturing conditions, as the frequency of M28z and MBBz CAR T cells with an exhausted phenotype was significantly higher following exposure to the HGSC cell line OVCAR-3 than SKOV-3.

In the orthotopic SKOV-3 model in **paper II**, progressive upregulation of exhaustion markers by M28z and MBBz CAR T cells was observed. Tumor-infiltrating M28z CAR T cells expressed higher levels of the PD-1 and TIM-3 exhaustion markers than MBBz CAR T cells. Furthermore, the MBBz-treated mice deemed under long-term remission displayed the least exhausted phenotype. We demonstrated that expression of exhaustion markers by MSLN-CAR T cells was dynamic and reversible in the absence of antigen stimulation in **paper I** and **II**. Following *ex vivo* resting in the absence of MSLN-antigen, certain mice-derived M28z and MBBz CAR T cells displayed cytolytic activity against MSLN+ target cells. Tumor-derived MBBz CAR T cells displayed higher *ex vivo* median lytic capacity than M28z CAR T cells, as well as increased effector cytokine production (IFN $\gamma$ , TNF, GrzB). Together these data imply that tumor-infiltrating MSLN-CAR T cells and in particular MBBz CAR T cells, are not terminally exhausted and can reinvigorate T cell functionality combined with the reversal of phenotypic exhaustion marker expression. CD28 and 4-1BB co-stimulation differentially impact the CAR T cell exhaustive state, and MBBz CAR T cells seem less prone to phenotypic exhaustion as well as exhaustion induced dysfunction.

Expression of the LAG-3 exhaustion marker by MSLN-CAR T cells was associated with detrimental CAR T cell functionality in **paper I** and **II**. More specifically, presence of PD-1/LAG-3/TIM-3 triple positive and LAG-3/TIM-3 double positive cell populations correlated with decreased target cell lysis in **paper I** and poor mice survival in **paper II** (**Figure 19A-B**). On the other hand, the simultaneous expression of PD-1 and TIM-3 was associated with enhanced CAR T cell functionality and prolonged mice survival.



**Figure 19. A)** Unbiased hierarchical clustering based on soluble plasma factors revealed cluster B and C, for which survival curves are displayed. **B)** Co-expression of CIMs by tumor-derived CD4+ CAR T cells of cluster B and C mice. Derived from **paper II**.

Our data underscore the detrimental impact of exhaustion on functional persistence of MSLN-CAR T cells *in vivo*. Importantly, MSLN-CAR T cells have the capacity to reinvigorate their effector functions in the absence of antigen stimulation. We identified the expression of LAG-3 in combination with TIM-3 alone or TIM-3 and PD-1 together as detrimental for functional

CAR T cell persistence. Together our data provide a window of opportunity for combinatorial treatment options for ovarian cancer, combining MSLN-CAR T cells with ICB beyond single blockade of PD-1. We speculate that ICB would augment the functional persistence of 4-1BB co-stimulated MSLN-CAR T cells in particular due to their enhanced capacity to restore functionality in the absence of antigen stimulation.

#### 4.4.3.2 Mice-derived M1xx CAR T cells displayed a less activated/exhausted phenotype

The expression of exhaustion markers by M1xx CAR T cells differed between *in vitro* and *in vivo* experiments in **paper III**. Following repeated *in vitro* stimulations M1xx CAR T cells displayed a more exhausted phenotype than M28z and MBBz CAR T cells, while mice-derived M28z and MBBz CAR T cells showed enhanced signs of exhaustion as compared to M1xx CAR T cells. In line with their phenotypic exhaustion profile, M1xx CAR T cells did not exert enhanced effector functions compared to M28z and MBBz CAR T cells *in vitro*, whereas M1xx CAR T cells elicited superior tumor control *in vivo*.

In the orthotopic SKOV-3 model, mice derived M1xx CAR T cells did not only display the least exhaustive phenotype, they also demonstrated superior functionality *ex vivo* upon re-stimulation as compared to M28z and MBBz CAR T cells. Recently, an NK-like functionally exhausted MSLN-CAR T cell population was described, characterized by expression of CD56 amongst others<sup>182</sup>. Tumor-derived CD8+ M28z and MBBz CAR T cells were mostly CD56+, while CD56 expression levels were absent/low in M1xx CAR T cells in **paper III**, confirming the less exhausted state of M1xx CAR T cells.

One important thing to keep in mind when interpreting these results is the disease status upon CAR analysis, with the M1xx-treated mice under long-term remission, whereas tumor progression was ruthless in M28z- and MBBz-treated mice. As previously described, expression of exhaustion markers is reversible and CAR T cells can recover T cell functionality in the absence of antigen stimulation. Our *in vitro* data demonstrated that M1xx CAR T cells can indeed display an exhausted phenotype and that exposure to a HGSC cell line *in vitro* can induce higher levels of exhaustion as compared to SKOV-3 cells. This raises the question whether the limited expression of exhaustion/activation markers (at the time of analysis) by of M1xx CAR T cells in the orthotopic SKOV-3 model is the result of calibrated CAR T cell activation and/or successful tumor clearance leading to absent/minimal antigen stimulation.

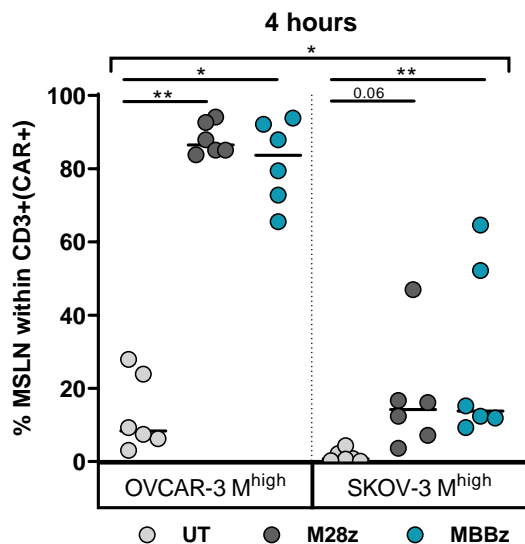
In the OVCAR-4 model in **paper III**, treatment with M1xx CAR T cells did not induce persistent tumor regression and mice therefore carried substantial tumor burden upon sacrifice. Due to the peritoneal disease spread of this model, we only analyzed spleen-derived CAR T cells. Spleen-derived M1xx CAR T cells were characterized by a naïve/memory phenotype, anti-apoptotic and self-renewal gene signature as well as specific upregulation of an internal T cell activation regulator, which has previously been linked to reduced expression of exhaustion markers such as PD-1<sup>183</sup>. This gene signature indicates that the tuned activation potential of M1xx CAR T cells was indeed responsible for the enhanced functional persistence *in vivo*, possibly by reduced induction of CIM expression amongst others. However, on a

transcriptional level exhaustion marker expression was comparable between spleen-derived M28z and M1xx CAR T cells and the cell surface CIM expression data are lacking for these specific MSLN-CAR T cells (OVCAR-4 model). Taken together we cannot exclude the possibility that the low levels of antigen stimulation contributed to the less activated/exhausted phenotype of M1xx CAR T cells in the *in vivo* SKOV-3 model nor can we exclude that immune control by M1xx CAR T cells eventually failed in the OVCAR-4 model due to exhaustion regardless of the persistence-promoting gene signature detected in spleen-derived M1xx CAR T cells compared to M28z CAR T cells.

Importantly, the M1xx CAR T cells studied in **paper III** contain a CD28 co-stimulatory domain (MSLN-CD28-1xx). Since 4-1BB co-stimulation shields MSLN-CAR T cells more from exhaustion than CD28 co-stimulation, it would be of great interest to develop and evaluate a MSLN-CAR construct containing calibrated activation potential and 4-1BB co-stimulation (MSLN-4-1BB-1xx). Based on our results we speculate that 4-1BB co-stimulated M1xx CAR T cells would display even greater functional persistence than MSLN-CD28-1xx CAR T cells as compared to classical second generation MSLN-CAR constructs.

#### 4.4.4 Trogocytosis as an immune escape mechanism

Throughout all papers we detected MSLN molecules on the cell surface of CAR T cells exposed to MSLN+ target cells both *in vitro* or *in vivo*. The MSLN antigen on the cell surface of target cells was nibbled off and ingested by CAR T cells, which subsequently cycled MSLN



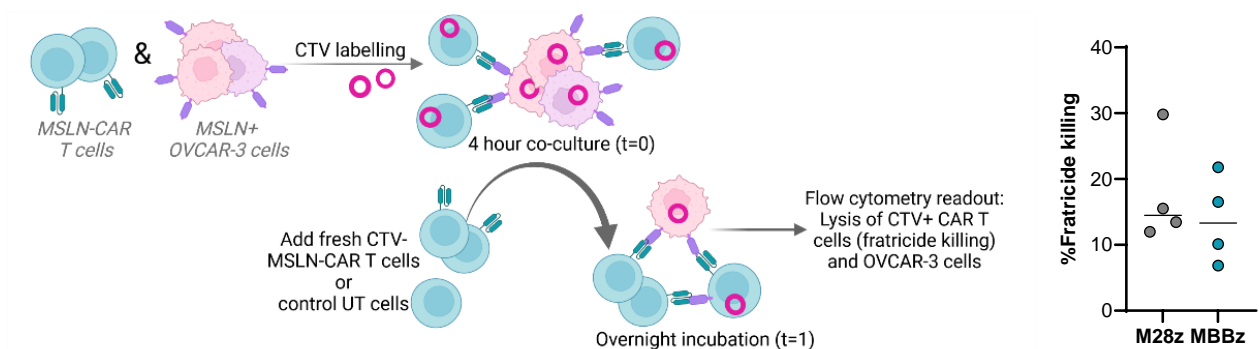
to the cell membrane for surface expression. All lymphocytes are capable of trogocytosis, however, in **paper I** and **II** we demonstrated that M28z- and MBBz-transduced T cells displayed superior trogocytotic capacity as compared to their non-transduced counterparts and untransduced T cells (**Figure 20**). These results imply that trogocytosis was largely CAR-mediated, thereby further limiting antigen availability on tumor cells for MSLN-CAR T cells.

**Figure 20.** MSLN expression within CAR+ M28z and MBBz CAR T cells (determined by anti-scFv staining) or untransduced T cells following four hours of co-culture with target cells. Derived from **paper I**.

M1xx CAR T cells were also demonstrated to have trogocytotic capacity *in vivo* in **paper III**, although the frequency of trogocytotic MSLN+ cells was lower in M1xx- than M28z- and MBBz-transduced CAR T cells. A prerequisite for trogocytosis is the presence of antigen-positive tumor cells and hence, the lower frequency of trogocytotic M1xx CAR T cells could be explained by superior tumor control and clearance of MSLN+ tumor cells and therefore the window of detection for trogocytotic CAR T cells was limited.

The degree of trogocytosis differed between *in vitro* cell culture conditions as the vast majority of M28z and MBBz CAR T cells was MSLN<sup>+</sup> following exposure to MSLN<sup>high</sup> OVCAR-3 cells while the frequency of MSLN<sup>+</sup> CAR T cells was lower following incubation with MSLN<sup>high</sup> SKOV-3 cells. The frequency of MSLN<sup>+</sup> CAR T cells mirrored the frequency of MSLN<sup>+</sup> tumors cells *in vitro*, as the level of trogocytotic CAR T cells was higher during the first hours of co-culture when MSLN<sup>+</sup> target cells were more prevalent. Linear regression analysis and unbiased hierarchical clustering demonstrated that the degree of trogocytosis was indeed dependent on the frequency of MSLN<sup>+</sup> target cells *in vitro*. More importantly, the presence of trogocytotic M28z and MBBz CAR T cells was negatively correlated with CAR T cell viability and lysis of target cells *in vitro*.

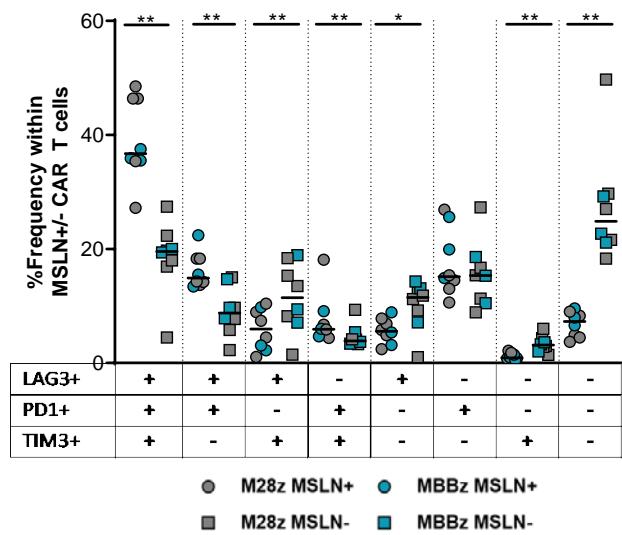
Trogocytosis by CAR T cells cuts as a double-edged sword, as it aids tumor antigen escape and diverts CAR T cells to killing of trogocytotic antigen-positive CAR T cells, also known as fratricide killing. In **paper I**, we demonstrated that the reduced viability of trogocytotic MSLN<sup>+</sup> CAR T cells can indeed be explained by fratricide killing (**Figure 21**).



**Figure 21.** Left: experimental set-up to assess fratricide killing of MSLN-CAR T cells and on the right, degree of fratricide killing. Derived from **paper I**.

Trogocytotic MSLN<sup>+</sup> M28z and MBBz CAR T cells were capable to reinvigorate proliferative capacity and effector functions, in the absence of antigen stimulation following short-term exposure to MSLN<sup>+</sup> target cells *in vitro*. However, these results do not exclude the possibility of trogocytosis-induced CAR T cell impairment following long-term or chronic antigen stimulation, as is the case *in vivo*. In **paper I** and **III**, trogocytotic MSLN<sup>+</sup> CAR T cells displayed a more exhausted phenotype compared to their MSLN<sup>-</sup> counterparts *in vitro* and *in vivo* (**Figure 22**), suggesting trogocytosis and CAR T cell exhaustion go hand in hand. The previously shown dynamic CIM expression has not been evaluated in the light of trogocytotic CAR T cells, and it would be of interest to investigate whether trogocytotic CAR T cells are more resistant to downregulation of exhaustion markers in the absence of antigen stimulation and reinvigoration of effector functions following immune checkpoint blockade.

Together these results underscore that CAR-mediated trogocytosis affects MSLN-CAR T cell functionality on several levels: i) tumor antigen escape ii) fratricide killing, and presumably, iii) T cell dysfunction through immunomodulation. As touched upon in the introduction,



trogocytosis can direct the fate of T helper subsets amongst others which would be of interest to investigate further.

**Figure 22.** Co-expression of CIMs within trogocytotic MSLN+ M28z and MBBz CAR T cells and their MSLN-counterparts. Derived from **paper I**.

## 5 CONCLUSIONS

In order to improve clinical response rates with MSLN-CAR T cell therapy for ovarian cancer, it is crucial to gain insights in the mechanisms behind functional CAR T cell persistence in relevant disease models. In this study, we demonstrated the potential and limitations of MSLN-CAR T cell therapy using several models of ovarian cancer. Each MSLN-CAR construct had its own signature, highlighting the important impact of CAR design on functionality.

All three MSLN-CAR constructs, M28z, MBBz and M1xx, showed potential for *in vivo* application as they significantly augmented survival of ovarian cancer-bearing mice. However, in the HGSC *in vivo* model, all mice eventually succumbed to disease progression despite initial anti-tumor responses elicited by MSLN-CAR T cells. Several aspects affecting functional persistence of MSLN-CAR T cells were addressed in **Paper I-III**, with each construct having its own signature, highlighting the importance of CAR design.

An overview of the concluding remarks:

- Bias towards the CD4<sup>+</sup> T cell population in the CAR<sup>+</sup> fraction following MBBz transduction, while CD4/CD8 ratio was comparable in M28z CAR T cells.
  - Skewness towards CD4<sup>+</sup> phenotype does not negatively impact cytotoxic potential of MBBz CAR T cells *in vivo* as compared to M28z CAR T cells due to efficient infiltration and/or expansion of CD8<sup>+</sup> MBBz CAR T cells.
- M28z and MBBz CAR T cells both effectively target MSLN<sup>high</sup> SKOV-3 and OVCAR-4 tumor cells as compared to control treatment, albeit with different kinetics.
  - M28z CAR T cells were more efficient in lysis of MSLN<sup>high</sup> SKOV-3 tumor spheroids and reached EC50 earlier than MBBz CAR T cells.
  - *In vivo* M28z CAR T cell treatment significantly delayed tumor progression, whereas MBBz CAR T cells were able to induce long-term remissions in certain cases.
- Calibration of MSLN-CAR T cell activation potential through mutations in the two distal ITAMs of the CD3ζ chain (M1xx) results in superior tumor control and functional CAR T cell persistence *in vivo*.
  - M1xx CAR T cell treatment resulted in 100% response rates (as determined by tumor regression below starting point) and persistent tumor control in an orthotopic model of ovarian cancer (SKOV-3).
  - In the setting of disseminated HGSC (OVCAR-4 i.p. model), M1xx CAR T cell treatment resulted in a survival advantage over M28z CAR T cell treatment, however all mice eventually succumbed to tumor burden.

- Several immune escape mechanisms were identified as detrimental to MSLN-CAR T cell functional persistence. Some immune escape mechanisms were universally shared between all three MSLN-CAR constructs, while the structure of CAR design impacted certain mechanisms differently.
  - Reduction in MSLN surface expression under MSLN-CAR T cell pressure *in vitro* and *in vivo*, aiding to antigen heterogeneity and antigen escape by tumor cells.
  - MSLN+ tumor cells specifically upregulate expression of co-inhibitory ligands under MSLN-CAR T cell pressure *in vivo*.
  - Upregulation of PD-1, LAG-3 and TIM-3 exhaustion markers by MSLN-CAR T cells during antigenic stimulation.
    - 4-1BB co-stimulated MSLN-CAR T cells less prone to phenotypic and functional exhaustion
  - MSLN-CAR T cells display trogocytotic capacity and trogocytosis negatively impacts successful anti-tumor responses by encouraging tumor antigen escape and fratricide killing.

## 6 POINTS OF PERSPECTIVE

Overall survival rates have not substantially improved for ovarian cancer patients in the past decades and ovarian cancer remains the 8th leading cause of cancer-related deaths among women globally<sup>6</sup>. The majority of patients are diagnosed with advanced ovarian cancer, characterized by widespread disease within the abdomen and approximately 70% of the patients succumb to the disease within 5 years<sup>2 7-9</sup>. These numbers underscore the need for novel therapeutic strategies for the treatment of advanced ovarian cancer. MSLN-CAR T cells are currently under investigation in various early stage clinical trials for the treatment of ovarian cancer. MSLN-CAR T cells demonstrated a good safety profile, however clinical response rates were marginal with limited functional persistence of CAR T cells in these studies. To improve clinical response rates with MSLN-CAR T cells, a better understanding of the mechanisms impacting CAR T cell functionality *in vitro* and *in vivo* is crucial. The work presented in this thesis underscores the potential and limitations, due to immune escape mechanisms, of MSLN-CAR T cells for clinical application in ovarian cancer.

Treatment with M28z, MBBz or M1xx CAR T cells significantly augmented survival in two different preclinical models of ovarian cancer, however, treatment outcome differed between construct. M28z CAR T cells elicited expeditious anti-tumor responses as compared to MBBz CAR T cells. Despite potent cytolytic activity, M28z CAR T cells were only capable of delaying tumor progression, whereas MBBz CAR T cells were capable of inducing long-term tumor control in SKOV-3 inoculated mice. M1xx CAR T cells elicited superior tumor control in the orthotopic SKOV-3 model, inducing long-term remissions. However, the SKOV-3 model does not represent HGSC and SKOV-3 cells are presumably of a different origin. HGSC is responsible for the vast majority of ovarian cancer related deaths and there is an urgent need for curative treatment options for these patients. The widespread micrometastases and unique TME niches of HGSC has proven an extremely challenging environment for immunotherapeutics. Even the powerhouse of M1xx CAR T cells were incapable of achieving long-term tumor control in the disseminated HGSC OVCAR-4 mice model and merely delayed disease progression. If we put the fight against cancer in the context of running a race with the first milestone being to reach the 10 km, the second to finish the half marathon and lastly to crush a full marathon, which advantage do our MSLN-CAR T cell constructs give ovarian cancer patients in running their race? M28z and MBBz CAR T cells are capable of pushing patients through the first 10 km and in rare cases, MBBz-treated patients can finish a half marathon. Treatment with M1xx CAR T cells can easily drive patients past the half marathon mark. However, M1xx CAR T cell treatment does not suffice in supporting patients to finish the full marathon of HGSC. Combinatorial treatment options are crucial to attain successful clinical response rates with MSLN-CAR T cells and for patients to finish this full marathon.

One of the major mechanisms through which ovarian tumors can avoid immune destruction is antigen heterogeneity already present upon start of treatment and CAR-mediated tumor antigen escape mechanisms including trogocytosis. This effectively entails that primary ovarian tumors and their widespread micrometastases contain antigen-positive and antigen-negative cancer

cells, It is of extreme interest to explore strategies targeting antigen-negative tumor cells. Induction of bystander killing has been proposed to overcome antigen heterogeneity, in which activation of CAR T cells by antigen-positive cancer cells can induce lysis of antigen-negative cancer cells through bystander mechanisms such as FAS and FAS ligand death receptor signaling, epitope spreading and secretion of soluble factors such as IFN $\gamma$  and TNF<sup>184-186</sup>. Despite the lack of demonstrated bystander killing by M28z and MBBz CAR T cells in our *in vitro* setting, we are hopeful for the induction of bystander mechanisms *in vivo*. Previous work by others has demonstrated the potential of MSLN-CAR T cell mediated bystander killing as well as the potential for combinatorial treatment options with cyclophosphamide and/or Birinipant to boost CAR-mediated bystander killing<sup>185-187</sup>. Another option that could be of interest to explore is the production of MSLN-CAR T cells from TILs and/or tumor-associated lymphocytes (TALs) found within solid ovarian cancer lesions and malignant ascites, respectively. The possible advantage of using TILs and TALs as a source for CAR T cells is their tumor homing capacity as well as their survival skills in the hostile ovarian cancer environment. More importantly, the non-transduced fraction of T cells are capable of targeting different antigens and in the case of TILs/TALs the frequency of tumor-specific cells is higher than in peripheral blood, allowing for successful epitope spreading and subsequent bystander killing<sup>52</sup>. Moving beyond bystander killing, dual and sequential antigen targeting has shown promising results in overcoming tumor antigen escape in a preclinical acute lymphoblastic leukemia model<sup>167</sup>.

The challenging environment of ovarian cancer, drives MSLN-CAR T cells towards exhaustion. We associated simultaneous expression of LAG-3/TIM-3 and PD-1/LAG-3/TIM-3 by M28z and MBBz CAR T cells with impaired functional persistence *in vitro* and *in vivo*. One pitfall of this thesis is the lack of exhaustion data on M1xx CAR T cells in the OVCAR-4 i.p. model, since now we can only assume that one of the reasons why mice relapsed is due to exhaustion induced CAR T cell dysfunction. This thesis provides a rationale for targeting LAG-3 with checkpoint blockade in order to reinvigorate MSLN-CAR T cell effector functions. Combinatorial treatment of LAG-3 and PD-1 has shown to provide a survival advantage compared to ICB of PD-1 alone in mice models of ovarian cancer as well as a phase II/III clinical trial in metastatic melanoma<sup>188-190</sup>. Combinatorial blockade of all three exhaustion markers poses an interesting alternative, as recent work demonstrated the potential benefit of triple blockade of PD-1, LAG-3 and TIM-3 over dual ICB targeting PD-1 and LAG-3 in a mouse model of ovarian cancer using HER2-directed CAR T cells<sup>191</sup>. Important to keep in mind is that adjuvant therapies focused on restoring CAR T cell functionality, do not impair the immunosuppressive capacity of the TME. Therefore, targeting different components of the TME such as CAFs and the vasculature amongst others is an interesting alternative strategy currently under evaluation in several studies.

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