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Immunotherapeutic targeting of tumor-associated macrophages

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ABSTRACT

The development of CAR T cell technology allows for MHC-independent recognition of a native target antigen by T cells. In august 2017, the first CD19-specific CAR T cell therapy was approved in the U.S. for the treatment of B-lymphoid malignancies. However, the translation into solid tumors has proven more challenging. Main obstacles include a paucity of appropriate tumor-specific antigens, and difficulties in facilitating CAR T cell migration to the tumor site and survival in the hostile microenvironment of the tumor.

Based on previous work from our group showing that re-polarization of tumor-associated macrophages (TAMs) by $CD4^+$ T cells can induce tumor regression, we hypothesized that CAR T cell targeting of TAM-specific antigens would lead to TAM re-polarization towards the tumoricidal phenotype with subsequent elimination of cancer cells. Given the abundance of macrophages in most solid tumors, this identifies a novel approach to cancer immunotherapy. In this study, we engineered a CAR construct specific for folate receptor beta (FRβ), a protein reported to be overexpressed that is overexpressed on TAMs, and investigated the use of FRβ-specific CAR T cells on TAMs *in vitro* and *in vivo* in established solid tumors. We found that CAR T binding activates and re-polarizes TAMs towards the pro-inflammatory and anti-tumor M1 phenotype, which are able to kill tumor cells *in vitro*. Re-educated TAMs secreted significantly higher amounts of pro-inflammatory cytokines and NO, and inhibition of iNOS activity completely abrogated macrophage-mediated killing, indicating that the anti-tumor effect triggered by FRβ-specific CAR T cells is dependent on iNOS activity *in vitro*. To fully assess the therapeutic potential of these CAR T cells, adoptive cell transfer experiments were performed in immunocompetent mouse tumor models. TAM-directed CAR T cell therapy slowed down tumor growth and led to regression of large, established tumors in models of melanoma and colon cancer and induced long-term remission in a subset of the treated animals.

Our findings highlight a novel approach to CAR T cell immunotherapy for solid tumors which is independent on the expression or secretion of tumor-specific antigens by the tumor cells, and which actively counteracts local immunosuppression at the tumor site.

ABBREVIATIONS

AA	Amino acids
ACT	Adoptive cell transfer
ADCC	Antibody-dependent cell-mediated cytotoxicity
AID	activation-induced cell death
AIF	Apoptosis-inducing factor
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukimia
APCs	Antigen presenting cells
ARG-1	Arginase
ATP	Adenosine triphosphate
BCR	B cell receptor
BSA	Bovine serum albumin
CD40L	CD40 ligand
CDC	Complement-dependent cytotoxicity
CLL	Chronic lymphocytic leukemia
cpm	Counts per minute
CRISPR	Clustered regulatory interspaced palindromic repeats
CRS	Cytokine release syndrome
CSCs	Cancer stem cells
CTL	Cytotoxic T lymphocyte
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
e.g.	For example
ECM	Extracellular matrix
EffLuc	Enhanced firefly luciferase
ER	Endoplasmic reticulum
Fab	Antigen-binding fragment
FasL	Fas ligand
Fc	Fragment crystallizable
FDA	Food and Drug Administration

FELASA	Federation of European Laboratory Animal Science Association
FRβ	Folate receptor beta
g	Gravitational force
(e)GFP	(enhanced) Green Fluorescent Protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HPSE	Heparanase
HSCs	Hematopoietic stem cells
i.d.	Intradermally
i.v.	Intravenously
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-1RA	IL-1 receptor agonist
iNOS	Inducible nitric oxide synthase
IRES	Internal ribosome entry site
LPSs	Lipopolysaccharides
M-CSF	Macrophage colony-stimulating factor
mAb	Monoclonal antibody
MCP-1	Monocyte chemoattractant protein-1
MCS	Multiple cloning site
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MMPs	Metalloproteinases
mRNA	Messenger ribonucleic acid
NIP	Hapten 4-hydroxy-3-iodo-5-nitrophenylacetic acid
NK cell	Natural killer cell
NKTs	Natural killer T cells
NO	Nitric oxide
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered Saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor

PGE2	Prostaglandin E2
PPRs	Pattern recognition receptors
RAG	Recombination-activation gene
rmFOLR2	Recombinant murine FRβ extracellular fragment
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
s.c.	Subcutaneously
scFv	Single-chain varibale fragment
SDF1	Stromal cell-derived factor
SPF	Special-pathogen free
STAT	Signal transducer and activator of transcription
TAMs	Tumor-associated macrophages
TCM	Tumor cell-conditioned medium
TCR	T cell receptor
T_{FH}	T follicular helper cells
TGF-β	Tumor growth factor beta
Th	Helper T cell
TILs	Tumor infiltrating lymphocytes
TLS	Tumor lysis syndrome
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
T_{regs}	T regulatory cells
U.S.	United States
VEGF	Vascular endothelial growth factor
VPA	Valproic acid
wt	Wild type

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Table **Page**

1 INTRODUCTION

1.1 The immune system

The word immunity stems from the Latin term *"immunitas",* and describes the ability of an organism to protect itself against infection and disease caused by exogenous or endogenous pathogens, while maintaining self-tolerance to prevent autoimmune diseases and hypersensitivity reactions (allergies). A perfectly coordinated balance between an active immune response and protection of normal tissue against damage is crucial [1].

The immune system is a complex network consisting of numerous different cell types and soluble molecules, each with specialized roles in the initiation, maintenance and regulation of the immune response. Based on the functional characteristics and evolutionary origin of the involved immune cell types, the immune system is subdivided into an innate/natural and adaptive/acquired arm, both working in a tightly interconnected manner to eliminate the antigen with humoral and cellular defense mechanisms. As the names indicate, the former is activated immediately after the presence of a foreign substance is detected, while the latter needs often some time (days to weeks) to fully develop, and is based on clonal expansion of antigen-specific lymphocytes [1, 2].

Innate immunity is a stereotypic, primitive, non-specific and universal response system without any inherent memory of past exposure to particular structures. Besides physical barriers and mechanisms like secretion of mucus, coughing and sneezing, innate immune cells are part of the first line of defense against invasion by pathogens. When a pathogen invades the body, bioactive molecules like acute phase reactants, the complement system, cytokines and effector cells such as natural killer (NK) cells, dendritic cells (DCs), granulocytes (basophiles/eosinophils/neutrophils) and macrophages will launch an attack against it. These are cell types with the ability to sense and respond to common molecular features of microbial agents and/or tissue injury. Mechanistically, for recognition of pathogens, the cells express pattern recognition receptors (PRRs) on their surface. This class of receptors either binds pathogen-associated molecular patterns (PAMPs), like components of the bacterial cell wall and bacterial lipopolysaccharides (LPSs), or damage-associated molecular patterns (DAMPs) like adenosine triphosphate (ATP) and biomolecules that are released following tissue injury, necrosis, cellular stress and/or malignant transformation of cells and tissues [3, 4].

The *adaptive immune response* is a highly specific and self-discriminating response system with memory development in the form of memory cells. It is composed of T and B lymphocytes, each having a unique antigen-specific receptor: T cell receptor (TCR) on T cells and B cell receptor (BCR) on B cells. The high specificity of this immune response is acquired by the broad repertoire of TCRs and BCRs which do not have their origin in the germline of an organism but are generated through genetic recombination (hypermutation) of the V(D)J gene segment. The generation of memory cells result in a faster and stronger secondary response to the same antigen [1, 2, 5].

1.2 T Lymphocytes

T lymphocytes, commonly referred to as T cells, develop from hematopoietic progenitors that migrate from the bone marrow into the thymus, where they undergo a highly coordinated maturation process. Once in the thymus, the V(D)J segment of the TCR gene undergoes multiple somatic mutations and rearrangements, resulting in formation of a TCR with a unique antigen specificity [6]. This process is the basis for the enormous heterogeneity of TCRs that underlies the ability of T cells to recognize virtually any antigen.

Before the generated T cells are released into the periphery, they must undergo a positive and negative selection in the cortex and medulla, respectively. This process is crucial to ensure a balance; the ability to productively recognize target antigens, against the risk of autoimmune damage resulting from recognition of self-antigens (structures normally present on healthy cells in the individual). *Positive selection* ensures that the generated TCRs are able to interact with the major histocompatibility complex (MHC) class I or II molecules expressed on cortical thymic epithelial cells, while autoreactive T cells are sorted out during *negative selection*. After the selection process the double positive T cells, expressing both the co-receptors CD4 and CD8 on its surface, downregulate in a random fashion either the co-receptor CD4 or CD8, thus becoming a naïve CD8 single positive T cell or a CD4 single positive T cells, respectively [7]. $CD4^+$ T cells and $CD8^+$ T cells are restricted to bind to processed antigens presented by MHC class II molecules and MHC class I molecules, respectively [8]. MHC class I molecules are expressed on the surface of nearly all nucleated cells, and present peptide fragments originated from intracellular 'house-keeping' proteins and in case of a viral infection viral proteins [9]. Functionally, MHC I presentation thus serves as a way of mirroring the composition of the intracellular proteome of the individual cell. MHC class II molecule

expression is limited to only a few so-called professional antigen presenting cells (APCs) like macrophages, DCs and B lymphocytes. MHC class II molecules present exogenous antigens from the extracellular milieu, like bacteria or phagocytosed dead cells, that have been taken up and processed [1]. MHC II presentation thus, predominantly provides a reflection of the antigenic composition of the microenvironment of the APC.

Binding of a naïve T cell to MHC class I or II molecules alone is not sufficient for its activation; a co-stimulatory signal is required. This signal is provided by co-stimulatory proteins on the APC, especially CD80/CD86 (B7-1 and B7-2), which interact with the CD28 receptor expressed on the T cell [10]. These co-stimulatory proteins are regulated by innate sensors of injury, including the PRRs. Thus, co-stimulation is only provided when the APC senses some sort of aberration of tissue homeostasis, ranging from physical trauma to infectious processes [11]. In this way, co-stimulatory molecules ensure that adaptive immune responses are appropriately triggered, and facilitate the integration of innate and adaptive immune responses. In the combined presence of TCR signaling and co-stimulation, the naïve T cell becomes activated and starts to proliferate, differentiate and acquires the ability to migrate to the site of infection or injury [10]. Naïve $CD4^+$ T cells, once activated, differentiate into subclasses of helper T (Th) cells with distinct effector functions [12]. While an increasing spectrum of Th subtypes have been described, the main classes include Th1, Th2, Th9 and Th17. These subsets have different effector functions, and are characterized by phenotypic features including the types of cytokines they produce, as further described in section 1.2.2.

Naïve $CD8^+$ T cells typically differentiate into cytotoxic T cells (CTL) following activation. They directly kill infected, abnormal and malignant cells that present a specific antigen on MHC class I molecules. This occurs in a contact-dependent and highly controlled manner. Target cell death may be triggered by numerous mechanisms, including secretion of cytokines, cytotoxic granules and Fas receptor/Fas ligand interactions, all resulting in induction of programmed cell death (apoptosis) [13-15].

1.2.1 T cell receptor (TCR)

During the 1980s, TCR encoding genes and their corresponding proteins were identified and characterized. This work revealed that TCRs exist as two classes of heterodimers, α/β TCR (95%), and the much less common γ/δ TCR [6, 16]. For the sake of simplicity and their

predominance in most anti-tumor immune responses, the focus of this work will be on α/β TCR.

The receptor, a heterodimer of α- and β -chains, consists of several domains. The extracellular domain resembles structurally a membrane bound antigen-binding (Fab) fragment of an antibody (immunoglobulin superfamily), including an antigen-specific variable (V) region followed by a constant (C) region. The V region is shaped during the thymic maturation process and confers the antigen specificity of the TCR. The C region is connected to a transmembrane domain spanning the membrane and ending as a short cytoplasmic tail. The two latter domains each associate with a zeta (ζ)-chain. For complete functionality, the core TCR interacts with the CD3 complex, consisting of CD3γ-, CD3δ, CD3ε-chain, via strong electrostatic interactions. Both the CD3 complex and the ζ-chains are needed for TCR stability and signal transmission to the cell's interior [6, 16-19]. Thus, the complete functional TCR complex is composed of eight subunits.

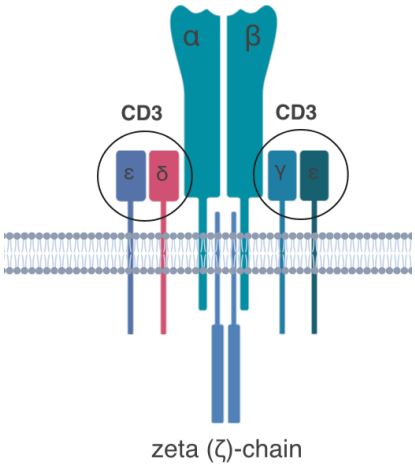

Figure 1. **Structural components of the T cell receptor (TCR).** The extracellular domain resembles structurally a membrane bound antigen-binding (Fab) fragment, which is connected to a transmembrane domain and ending as a short cytoplasmic tail, collectively referred to as the core complex. The transmembrane domain and cytoplasmic tail each interact with zeta (ζ) chains. For full functionality, the core complex associates with the CD3 complex. (Illustration was made by using components from BioRender)

1.2.2 CD4⁺ T cells

The CD4⁺ T helper cells are key players in adaptive immunity, as they are involved in initiating, shaping and maintaining both B- and T-cell mediated immune responses. They help to activate an immune response through release of cytokines and direct interaction with other immune cells such as macrophages and B cells, as well as other T cells [12].

Differentiation of $CD4^+$ T cells into different subsets, each with distinct effector functions, enables target specific, effective and regulated immune responses. Upon binding of naïve $CD4^+$ T cells to antigen-MHC class II complexes on APCs, cytokines secreted by the APCs direct the differentiation of the T cell into one of the different effector cell types, thereby determining what type of immune response is elicited. Subclasses of effector T helper cells are classified according to the cytokines inducing the pathway of differentiation, the activated lineage-specific transcription factor, the cytokine secretion profile and effector functions. The first subsets characterized by Mosmann et al. [20] were T-helper 1 (Th1) and T-helper 2 (Th2) cells, with identification of further subsets in the course of the last decades: T regulatory cells (T_{regs}), T follicular helper (T_{FH}) cells, Th9 and Th17 cells [21]. The qualitative nature of a $CD4^+$ T cell response is largely shaped by the dominating Th subset. Characterization of the Th polarization is therefore informative in understanding the nature and functional consequences of an adaptive immune response. T_{regs} are a functionally distinct subset of $CD4^+$ T cells that provides a negative feedback in immune responses, by dampening adaptive immune responses and inhibiting effector T cell functions, as further elaborated below [1].

Th1 cell differentiation is triggered by interleukin (IL)-12 and interferon gamma (IFN-γ), which is released upon intracellular bacterial- and viral infections [1, 22]. IL-12 and IFN-γ activate STAT4 (signal transducer and activator of transcription) and STAT1, respectively, followed by expression of the transcription factor T-bet [23]. Activated Th1 cells are responsible for a cell mediated and inflammatory immune responses through acting on B cells, $CD8^+$ T cells and macrophages and also play important roles in tumor cell elimination [22, 24]. Secretion of IFN-γ causes classical macrophage activation [25]. Stimulated macrophages show increased phagocytosis activity and production of free radicals that all result in killing of extracellular pathogens. Other effector cytokines secreted are IL-2 and tumor necrosis factor (TNF)-beta [1, 26].

Th2 cells dominate in responses against extracellular parasites, such as helminths, orchestrate humoral and non-inflammatory immune responses, and promote fibrosis/repair of damaged tissue [1, 27]. IL-4 drives the T cell into a Th2 cell through activation of STAT6, followed by expression of the master regulator transcription factor GATA3 [28]. Effector cytokines secreted include IL-4, IL-5, IL-13 and IL-10 [1, 29, 30]. IL-4 is pivotal for Th2 differentiation and

stimulates B cells to produce immunoglobulin (Ig) E antibodies with subsequent stimulation of mast cells [31]. IL-5 activates eosinophils and IL-10 suppresses Th1 driven immune responses through inhibition of macrophage activation [1, 29, 32].

Th17 cell polarization typically occurs in the combined presence of IL-6, tumor growth factor beta (TGF-β), IL-23 and IL-21, which activate the master transcription factor RORγT [33, 34]. The cytokine characteristically secreted by *Th17 cell*s is IL-17 [35]. These cells play a role in immune responses against extracellular bacterial infections by recruiting neutrophils and macrophages to the site of inflammation [1, 33, 36].

T_{regs} are not involved in generation of immune responses against pathogens, but regulate and suppress ongoing T cell responses through several mechanism. These cells are crucial in maintaining immune tolerance and tissue homeostasis under normal conditions, limit the extent and duration of inflammatory responses, and promote healing following inflammatory injury [1]. In the context of cancer, these cells appear to play important roles in the suppression of spontaneous and therapeutic antitumor immune responses [37]. T_{reg} polarization is induced by TGF-β but in the absence of IL-6. Effector cytokines are TGF-β and IL-10 [1, 38].

It is now apparent that $CD4^+$ T cells have a higher degree of functional plasticity than previously considered [39]. Findings suggest that fully differentiated and functional $CD4^+$ Th cells, including Th17 and T_{regs}, are capable of switching their phenotype to another Th subset [39, 40]. This plasticity is likely to play important roles in balancing effector T cell and T_{regs} during an immune response. It can also be utilized therapeutically to reshape the nature of adaptive immune responses in various diseases, including autoimmunity, infectious diseases, degenerative diseases and cancer [39, 41].

1.3 Macrophages (Mϕ)

Macrophages are large mononuclear phagocytes derived from monocytes that originate from bone marrow hematopoietic stem cells (HSCs). They are specialized in detection and phagocytosis of old/damaged/transformed cells, cell debris and (products of) microorganisms with following destruction. They are considered to be the body's first line of defense. Besides

their key roles in the immune system, both during innate and adaptive immune responses, macrophages are also involved in tissue homeostasis, wound healing, angiogenesis and muscle regeneration [42, 43]. Their cell shape is highly variable, and the cell interior contains numerous vacuoles and lysosomes. The cell surface bears distinct PRRs, fragment crystallizable (Fc)-receptors and complement receptors for binding of pathogens or opsonin (e.g. antibodies, complement proteins C3b/C4b/C1q, circulating proteins collectins/ficolins, pentraxins) that are marking the target cell [1]. Moreover, macrophages are a highly plastic cell type that can change their functional phenotype depending on exposure to different stimuli. A simplified, dichotomous scheme classifies macrophages into classically activated (M1) and alternatively activated (M2) macrophages. These polarization profiles likely represent two extremes of a broad and complex spectrum of macrophage phenotypes [44, 45]. While artificial and incomplete, this dichotomy provides an easy and convenient framework for understanding the role of macrophages in various contexts, including cancer. Thus, in the following, I have chosen to apply the terms M1-like and M2-like macrophages to refer to the pro-inflammatory and anti-inflammatory macrophage phenotypes of tumor-infiltrating macrophages, respectively.

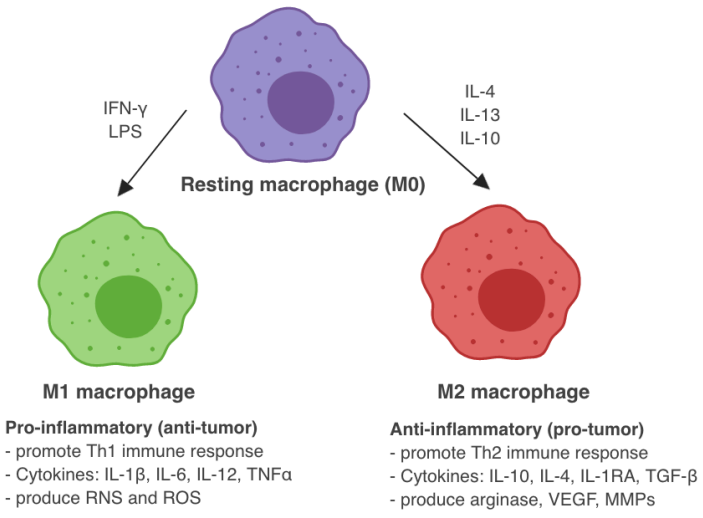

Figure 2. **Macrophage polarization and effector functions.** Classical activated M1 macrophages promote a Th1 driven immune response against pathogens and tumor cells. Pro-inflammatory cytokines secreted include IL-1β, IL-6, IL-12, TNF-α, as well as reactive nitrogen species (RNS) and reactive oxygen species (ROS) are produced. Alternatively activated (M2) macrophages promote an anti-inflammatory Th2 driven immune response and tumor progression. They play key roles during resolution of an immune response, and are involved in tissue remodeling, angiogenesis and tissue repair. Cytokines secreted include IL-10, IL-4, IL-1RA. IFN-γ, interferon-gamma; LPS, lipopolysaccharide; IL, interleukin; TNF-α, tumor necrosis factor alpha; RNS, reactive nitrogen species; Th, T helper cell; TFG-β, transforming growth factor beta; IL-1RA, interleukin 1 receptor agonist; VEGF, vascular endothelial growth factor; MMPs, metalloproteinases. (Illustration was made by using components from BioRender)

1.3.1 Classically activated macrophages (M1)

Macrophages exposed to the bacterial component LPS in combination with IFN-γ derived from immune cells like NK cells and Th1 T cells, will polarize towards the pro-inflammatory and anti-tumor phenotype (M1) [46]. Moreover, binding of CD40 ligand on Th1 cells to the CD40 receptor on macrophages has also shown to direct the macrophage into a M1-like state. Once activated, macrophages increase expression of MHC class II molecules and co-stimulatory ligands (CD80, CD86, CD40) and secrete various cytokines like IL-12/IL-18, thereby promoting bidirectional activation and polarization of resting $CD4^+$ T cells into Th1 cells. Hence, M1-like macrophages promote a Th1 driven immune response [46-48].

Besides their ability to phagocytize pathogens and other harmful particles, activated M1 macrophages secrete a cocktail of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12 and TNF-α, as well as reactive nitrogen species (RNS), reactive oxygen species (ROS) and proteases with great potential to eliminate pathogens and tumor cells. If secretion is not highly regulated, this can lead to great tissue damage [46]. It has also been shown that increased distribution and infiltration rate of M1-like macrophages seems to have a positive prognostic impact on survival rate of a patient [49-51].

1.3.2 Alternatively activated macrophages (M2)

When macrophages are exposed to IL-4 alone or in combination with IL-13 and/or IL-10, secreted by Th2 cells, basophiles or macrophages themselves, polarization is driven towards an anti-inflammatory M2-like phenotype. M2 macrophages are further divided into several subgroups characterized by cytokines, immune complexes (e.g. soluble antigen-binding antibodies) and pathogen particles that drive the polarization [52]. M2 macrophages, in contrast to M1-like macrophages, display a pro-tumor and immune-suppressive phenotype. They play key roles during resolution of an immune response, inflammation, remodeling processes, angiogenesis and orchestrate tissue repair (wound healing). They are also involved in the development of an allergic inflammation and have been shown to promote tumor progression [53, 54].

M2 macrophages produce, among others: arginase (ARG-1), the anti-inflammatory cytokine IL-10, the IL-1 receptor antagonist (IL-1RA), vascular endothelial growth factor (VEGF) and metalloproteinases (MMPs). The two latter take part in remodeling the extracellular matrix [53, 55-58].

1.3.3 Tumor-associated macrophages (TAMs)

The tumor microenvironment of solid tumors is comprised of a large number of different cell types and molecules with important functions for establishment of an environment that favors tumor growth and progression, through formation of new blood vessels and repression of a host's immune response. One of the major immune cells infiltrating the tumor are macrophages, in this context commonly referred to as tumor-associated macrophages (TAMs). Tumor cells and other cells in the tumor mass secrete, among others, monocyte chemoattractant protein-1 (MCP1), macrophage colony-stimulating factor (M-CSF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and various other chemokines that actively recruit circulating monocytes to the tumor site [59-61]. At the tumor site, monocytes differentiate and polarize into a tumor promoting M2-like phenotype in response to cytokines like IL-3, IL-4, IL-10, TGF-β released by tumor cells, tumor-infiltrating T and B cells, TAMs and other stromal cells, but also in response to various other factors like extracellular matrix (ECM) components, stromal cell-derived factor 1 (SDF1), macrophage migration inhibitory factor (MIF) and prostaglandin E2 (PGE2) [59, 62, 63].

TAMs display a tumor promoting and anti-inflammatory phenotype. They play crucial roles in tumor cell survival, tumor growth and progression. They silence adaptive immune responses driven by Th1 cells, promote neoangiogenesis to ensure sufficient nutrient supply within the growing tumor mass and modify the ECM. All these functions are achieved by releasing growth factors, chemokines, cytokines (e.g. IL-10), substrates/molecules, proteases and production of arginase [59].

Furthermore, TAMs can, in some cases, make up to 50% of the tumor mass and their infiltration rate has shown to correlate with poor survival prognosis of a patient. This has been reported for several different cancers including breast cancer, lung cancer and renal cell carcinoma [59, 64-66].

In addition, macrophages are able to switch their phenotype in response to different environmental factors/cues. Tumor infiltrating macrophages and already existing macrophages that have been educated towards an M2-like phenotype by the tumor environment can be re-polarized towards an anti-tumor M1-like phenotype and hence change their effector functions at the tumor site dramatically [67, 68].

Since tumors with a high rate of TAMs demonstrate a higher resistance against chemotherapy and radiotherapy, TAMs become more and more important as a potential therapeutic target

for immunotherapy. There are several strategies to alter the behavior and function of TAMs: inhibiting the recruitment and infiltration of monocytes/macrophages to the tumor site, blocking of TAM effector functions, depletion of TAMs from the tumors and re-polarization of TAMs to an anti-tumor phenotype (M1) [68-70]. Re-polarization is achieved by either using Th1 cells to interact with macrophages and thus induce re-polarization or by using antibodies that bind to the co-stimulatory receptor CD40 (agonistic anti-CD40 monoclonal antibody (mAb)), thereby mimicking the interaction between a macrophage and T cell [71, 72].

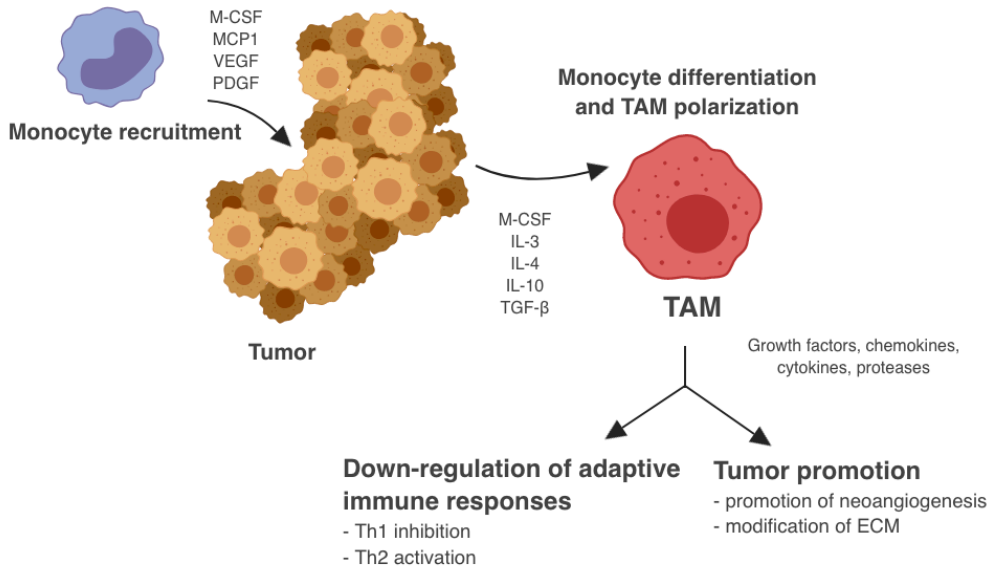

Figure 3. **Overview of monocyte recruitment and differentiation into TAMs, and their functional involvement in tumor progression.** Monocytes are recruited to the tumor. At the tumor site, monocytes differentiate and polarize towards mature M2-like macrophages, now referred to as TAMs. They play pivotal rules in tumor promotion and down-regulation of adaptive immune responses. Adaptive immune responses are silenced by inhibiting Th1 cells and recruiting Th2 cells and T_{regs}. Tumor promotion is facilitated by producing molecules needed for tumor cell survival, ECM remodeling and neoangiogenesis, which ensures sufficient nutrient supply for tumor cells. M-CSF, colony stimulating factor 1; MCP1, monocyte chemoattractant protein-1; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; IL, interleukin; TGF-β, transforming growth factor-beta; Th, T helper cell; TAM, tumor associated macrophage; ECM, extracellular matrix. (Illustration was made by using components from BioRender.)

1.4 Tumor immunology

Understanding the interaction between tumor cells and the host immune system is important for development of new immunotherapeutic treatment strategies. Malignant cells are the results of numerous acquired genetic mutations resulting in alterations in gene expression pattern, proliferation rate and behavior. In 1909, Paul Ehrlich was the first to formulate a hypothesis stating that malignant cells are recognized and eliminated by the immune system [73]. Around 50 years later, this hypothesis, referred to as the immunosurveillance hypothesis, was further refined by Burnet and Thomas, proposing the role of (especially) lymphocytes in recognizing and attacking malignant/transformed cells [74-76]. Due to technical as well as conceptual limitations, such as the inability to culture T cells *in vitro* and the fact that tumor-specific antigens were not yet identified, the immunosurveillance hypothesis gained little acceptance. A growing understanding of the immune system, with mounting experimental data from both humans and mice, it was later demonstrated that the lack of a functional immune system was associated with increased development of malignancies, leading to a broader acceptance of the immunosurveillance hypothesis [76]. Here, the development of mice deficient in the recombination-activation gene (RAG), a gene involved in V(D)J recombination, thus lacking functional B and T lymphocytes, allowed direct demonstration of a relationship between immunodeficiency and higher risk of developing cancer [77]. Further evidence was gained by reports stating that mice lacking key effectors of adaptive immunity, including perforin-deficient mice, as well as mice lacking functional IFN-γ signaling, showed an increased susceptibility to cancer development upon carcinogen exposure [78-80].

However, data presented by Shankaran et al. (2001), showed that wild type immunocompetent mice were able to develop cancer upon transplantation of a sarcoma from ordinary immunocompetent mice [79]. These findings indicated that, besides being able to detect and eliminate cancerous cells, the host immune system shapes the immunogenicity of tumor cells by imposing a selection pressure during tumorigenesis that promotes the outgrowth of tumor cells with the ability to avoid or overcome recognition by the immune system. Consequently, the concept of tumor surveillance was redefined and extended into the concept of tumor immunoediting. According to this concept, immunoediting of transformed cells by the host's immune system occurs through three phases: elimination, equilibrium, escape [79, 81, 82].

The *Elimination-phase* is the phase in which the host's immune system detects and eliminates transformed (malignant) cells at an early stage of tumor formation. At this early stage, both tumor cells and cells surrounding the tumor secrete and express stress-induced molecules (e.g. tumor antigens, DAMPs, inflammatory cytokines). This leads to an activation of immune cells from both the innate and adaptive immune response (NK cells, DCs, macrophages, $CD4^+$ and $CD8^+$ T cells), which in turn limit proliferation and expansion of the incipient tumor. Tumor cells that are able to withstand this immune attack can enter the *equilibrium-phase*. During this phase (which can last for many years), tumor cells are under constant control by the host's immune system ensuring that the remaining and faster proliferating tumor cells cannot expand or spread, meaning that the cancer cells are held in a functional dormant state. However, the immune system is not capable of fully eliminating these residual (less immunogenic) tumor cells. Only cells that have acquired mutations allowing them to evade and escape from the immune system are able to resume expansive growth and enter the third phase, referred to as *escape-phase*. Once tumor cells enter this phase, they can proliferate and expand in a more unrestricted manner, since they have now developed several mechanisms to evade the immune system. Besides having gained mutations in oncogenes and tumor suppressor genes that allow uncontrolled cell division, other mechanism include, among others, loss of immunogenic tumor-specific antigens, expression of immune inhibitory receptors (e.g. PD-L1) on their surface, secretion of immunosuppressive factors and cytokines that blunt or halt immune responses thereby leading to the formation of an immunosuppressive tumor microenvironment [83-85].

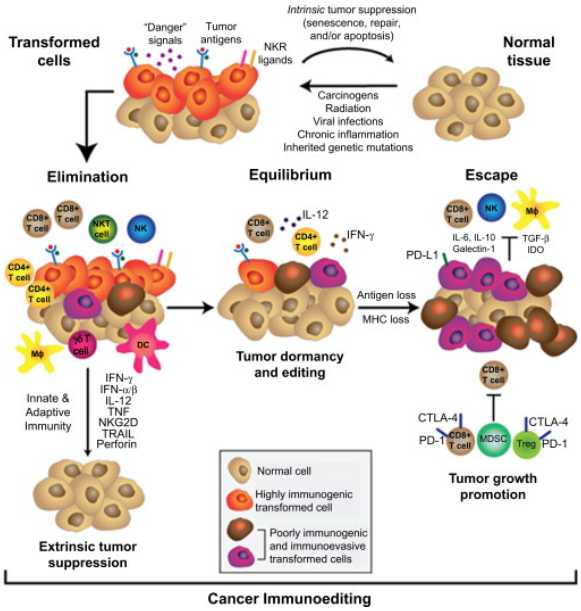

Figure 4. **The concept of immune tumor editing.** Immunoediting of transformed cells by the host's immune system occurs through three phases: elimination, equilibrium, escape. **Elimination phase:** Transformed (malignant) cells are detected and eliminated by both innate and adaptive immune responses at an early stage, before they become clinically apparent. Tumor cells that are able to withstand this immune attack can enter equilibrium. **Equilibrium-phase:** Tumor cells are under constant control. The immune system ensures that the transformed cells remain in a functional dormant state. However, the immune system is not capable of fully eliminating these residual tumor cells. Cells that have acquired mutations allowing them to evade the immune system are able to enter the third phase. **Escape phase:** Tumor cells that enter this phase can proliferate and expand in an immunological unrestricted manner. (Figure is reprinted from [84].)

1.4.1 Immunotherapy

Cancer is a very heterogenous disease. Key elements including cancer type, its location in the body, grade of the tumor, and also the general health stage of the patient play important roles in defining the appropriate treatment method. Most often, a combination of different therapeutic modalities is used to attack the tumor. Besides surgery, chemotherapy, radiation therapy, hormone therapy, more and more forms of targeted therapies, including the use of small-molecular-weight enzyme inhibitors and tumor-specific mAb therapy have become available in the past 20 years. mAb therapy can be considered the first class of therapeutics utilizing components of the immune system, now referred to as cancer immunotherapy.

Immunotherapy is based on the idea of stimulating the immune system to easier recognize, target and eliminate cancer cells, either by enhancing the patient's own immune cells, or by initiation of artificial *de novo* immune responses [86]. The unmatched specificity of immune cell recognition provides the potential for highly targeted attacks of malignant cells, and a fundamentally different mode of action makes it a potential salvage option for patients that have shown resistance against traditional treatment modalities such as radiation and chemotherapy. Different approaches are utilized, with an increasing spectrum of treatment

strategies. As previously mentioned, the classic form of immunotherapy is the use of mAbs to target cell surface molecules on the cancer cell. This can be used to make the cell detectable for other immune cells (non-specific immune stimulation), which may trigger natural occurring processes such as antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). mAbs can also be used for targeted delivery of cytotoxic agents, including cytotoxic drugs or radioactive isotopes, or to block immunosuppressive signals (e.g. immune checkpoint inhibitors) [87]. Other strategies include the use of vaccines, cytokines, oncolytic virus and cellular therapies involving active transfer of immune cells (adoptive cell transfer; ACT). This latter class of immunotherapy, which predominantly utilizes T cells, is the subject of the current thesis, and will be further addressed in the sections below [86, 87].

1.4.2 T cells in immunotherapy

Playing a critical role during tumor surveillance and tumor editing, immune cells in general, and T cells in particular, have received increasing focus as potentially powerful tools in cancer treatment.

Different strategies are used to enhance or trigger tumor-specific T cell responses. Administration of antibodies, cytokines and checkpoint inhibitors have shown to prolong T cell survival and enhance infiltration rates of T cells [88, 89]. Fundamental to such strategies is the thought that patients harbor tumor-specific T cells with the ability to mediate clinically meaningful attacks of the tumor cells through interventions that enhance their potency. Cancer vaccines targeting $CD4^+$ and $CD8^+$ T cells have been developed, with the aim to induce *de novo* T cell responses against tumor antigens [90-92]. Yet another strategy, ACT of autologous or allogeneic $CD4^+$ and $CD8^+$ T cells is developing, which is based on boosting the immune system by infusion of naturally occurring or engineered tumor-specific T cells that have been artificially expanded to large numbers outside the body. This strategy is addressing the problem that a key limitation in natural anti-tumor immunity is a failure to spontaneously achieve the required amount of functional effector T cells. ACT therapy has demonstrated promising results in several clinical trials [93]. In 1988, the first T cell therapy recognizing a specific cancer antigen was used for the treatment of metastatic melanoma. Tumor infiltrating lymphocytes (TILs) were extracted from a tumor and cultured in medium containing IL-2. Selected TILs, showing anti-tumor activity, were further expanded and then transferred back into the patient [94]. However, the duration and persistence of transferred cells was limited.

This problem was solved by depletion of the host's own lymphocytes by either chemotherapy alone, or in combination with whole body irradiation of the patient prior to TIL-ACT [95]. In addition to TIL-based immunotherapy, ACT of genetically engineered cancer-specific T cells, including TCR-transduced T cells and chimeric antigen receptor transduced T cells (CAR T) have been developed [96, 97]. ACT is a promising and personalized approach for cancer immunotherapy, and may provide novel opportunities for precision therapy in patients with previously incurable cancers.

1.4.3 Chimeric antigen receptor (CAR) T cells

The need for antigen recognition in the context of MHC molecules complicates the therapeutic use of T cells, since an individual TCR will only be applicable in MHC haplotype-matched individuals. Similarly, determining the specificity of a T cell is difficult, since its target antigen is presented as a small peptide bound to an MHC molecule, thus making it necessary to study the entire MHC/antigen complexes. This contrasts B cell receptors/antibodies, where the target is a freely presented intact antigen that is recognized in its native state, and hence easier to identify [1]. Chimeric antigen receptors (CARs) represents a class of synthetic T cell receptors that allows MHC-independent recognition of a native target antigen by T cells [98]. The development of CAR T-cell therapy represents a new paradigm in immunotherapy.

In 1989, Eshhar et al. were the first to describe the concept of CAR T cells [98]. They engineered a chimeric T cell receptor with the aim to direct specificity of the T cell towards an antigen (e.g. tumor antigen) of choice that was not dependent on being expressed on an MHC molecule [98]. In essence, a CAR combines the antigen recognition capabilities of a B cell receptor, while utilizing the intracellular signaling apparatus of the T cell. Composition of the extracellular domains and the intracellular signal components determine binding affinity, overall effector functions and behavior of the T cell and needs to be adjusted for the intended purpose [99-101].

The CAR is a single polypeptide chain with an extracellular antigen-binding site comprising the heavy chain variable region and a light chain variable region of an antibody with a defined target specificity. This so-called single chain variable fragment (scF_V) is fused to a transmembrane region that anchors the receptor to the cell membrane, and connects the scF_V with an intracellular part (cytoplasmic tail). The cytoplasmic tail contains one or more signaling domain(s) that trigger TCR signaling [102]. The fundamental component in most CARs is the

CD3 ζ-chain signaling domain, which is the central signal transduction part of a physiological TCR signaling complex. This domain triggers the basic activation process that occurs upon binding of a TCR to its complex [102-104]. Addition of one or more signal transduction domain(s), most commonly derived from the co-stimulatory receptors CD28 or 4-1BB, OX40 and CD27, provides additional signals that further activate key T cell effector functions, and protects the cell against apoptosis. This has been found to be essential in promoting an effective and long-lived activation of the CAR T cells that shows an increase of efficiency in therapeutic settings [99, 102]. By linking signaling and co-stimulatory domains in one polypeptide chain, CAR T cells are capable of getting activated only upon binding to its specific target [105]. CAR T cells harboring only the CD3ζ domain are referred to as 1st generation CAR T, addition of one co-stimulatory domain yields a 2nd generation CAR T, while combinations of two co-stimulatory domains yields 3rd generation CAR T. The structure of the CAR T greatly impacts the quality (in terms of activation and proliferation), the magnitude of IL-2 secretion, and cytotoxic effector functions [99]. A 4th generation of CARs ("armored CARs") have been developed. In addition to the CAR, these cells express a gene (e.g. cytokine transgenes like IL-12, IL-15). The additional cytokine secretion provides extra signaling aimed at improving effector activity by supporting expansion and longevity of the CAR T in an autocrine fashion, and may also conceivably work in a paracrine manner by effects on other immune cells present within the tumor environment [99, 106, 107]. Data from preclinical models support the notion that armored CAR T cells have improved longevity and therapeutic efficacy [108]. With CAR T cell technology, it is theoretically possible to target any molecule (independent of being expressed by MHC complex) on a malignant cell or any other cell present in the tumor microenvironment resulting in direct or indirect elimination of the tumor cell. Furthermore, it enables a more non-personalized immunotherapy since the same CAR construct can be used for many different patients.

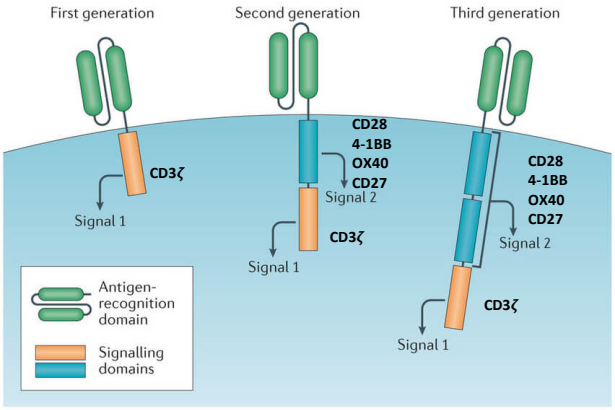

Figure 5. **Schematic illustration of the chimeric antigen receptor (CAR).** The CAR is a single polypeptide chain with an extracellular antigen-binding site connected with an intracellular part via a transmembrane domain. The cytoplasmic tail contains one or more signaling domains that trigger TCR signaling. CAR T cells harboring only the CD3ζ domain are referred to as 1st generation CAR T, addition of one co-stimulatory domain yields a 2nd generation CAR T, while combinations of two co-stimulatory domains yields 3rd generation CAR T. (Figure is modified from [109])

1.4.4 Adoptive cell transfer of CAR T cells

Adoptive transfer of lymphocytes is an evolving strategy to redirect T cells towards a specific antigen, such as tumor antigens. For this, white blood cells are collected from the patient's blood by leukapheresis and lymphocytes isolated. Next, the gene encoding the engineered CAR is introduced into the T cells *in vitro*, and CAR expressing T cells are then expanded *in vitro* before they are infused back into the patient [110]. Different approaches are used to introduce the gene. Traditional approaches include viral-mediated transduction (lenti- or retroviral) and nonviral gene transfer of deoxyribonucleic acid (DNA) plasmids and messenger ribonucleic acid (mRNA) species (by utilizing e.g. transposon elements or electroporation) [111-113]. These approaches do not result in disruption of the resident TCR gene thus making it necessary to only use autologous lymphocytes; otherwise there would be a risk of rejection of injected T cells or development of a graft-versus-host disease [112]. Eyquem et al. have recently developed a strategy that could overcome this issue [114]. Using the clustered regulatory interspaced palindromic repeats (CRISPR)/Cas9 technology (further described in section 1.5), a site directed insertion of the CAR encoding gene is ensured, as well as it can be used to disrupt TCR encoding genes at the same time as the CAR encoding gene is introduced [114]. To enhance engraftment, expansion, persistence and efficiency of adoptively transferred T cells, host immunodepletion with radiotherapy or chemotherapy prior to ACT is crucial. Depletion of lymphocytes and other immune cells (also resulting in decrease of cytokine levels) creates homeostatic space for the CAR T cells which facilitates their survival and functionality in the host [115].

In the past few years, ACT of CD19-specific CAR T cells has shown impressive results in the treatment of B-lymphoid malignancies, especially for chronic lymphocytic leukemia (CLL) and acute lymphoblastic leukemia (ALL) [116-120]. In august 2017, the first CAR T cell therapy (Kymriah™) was approved by the Food and Drug Administration (FDA) in the United States (U.S), and two additional CD19 CAR T cell therapies have later been approved for clinical use.

Side effects of CAR T cell therapy

CD19 CAR T cell immunotherapy has an impressive therapeutic potency, but in its current form, it comes with the price of potentially life-threatening side effects, largely related to the massive and widespread T cell activation that occurs upon target binding [121]. B cell aplasia is a common seen side effect, which can lead to hypogammaglobulinemia, a condition of decreased antibody production, thus being potential life-threatening for the patient [121, 122]. Moreover, recognition and depletion of normal immune- and tissue cells expressing the antigen recognized by the CAR T cell can cause severe tissue damage and in extreme situations lead to death [121, 123, 124]. To avoid unexpected cross-reactivity, it is crucial to perform extensive studies examining expression levels of the CAR-specific antigen on normal tissue during the phase of CAR development. Other distinctive and problematic side effects associated with CAR T cell therapy are the cytokine release syndrome (CRS) and tumor lysis syndrome (TLS) [115, 125-128], as well as transient and fully reversible neurologic toxicities, including loss of balance, severe headaches, confusion and speech difficulties [121, 129].

CRS is a systemic condition, in which a massive release of cytokines into the blood stream is detected. These cytokines are either directly secreted by adoptively transferred CAR T cells or other immune cells that are activated upon direct T cell-immune cell interaction. Cytokines including IL-2, IL-6, IFN-γ will affect the normal health state of a wide variety of organs. Furthermore, severity of CRS has been graded based on cytokine profiles. Administration of an anti-IL-6 receptor antibody (tocilizumab) has been shown to reduce CRS toxicity efficiently and was in august 2017 approved by the FDA in the U.S for treatment as CAR T cell therapy induced CRS [115, 130, 131]. On the other hand, TLS occurs when a large number of tumor cells are lysed at the same time, which results in release of their intracellular content into the bloodstream thereby changing the electrolyte level and cause metabolic abnormalities. These changes result in vomiting and nausea as well as more life-threatening conditions including kidney- and renal failure, seizures and cardiac arrhythmias [115].

1.4.5 Activated Th1 CD4$^+$ T cells induce macrophage activation

Activation and polarization of macrophages into different phenotypes, and their ability to switch towards another phenotype (re-polarization) upon encountering specific stimuli is now well documented. Yet, the exact molecular basis of macrophage polarization is incompletely understood. *In vitro,* IFN-γ together with a second signal such as LPS, drive the macrophage towards an M1-like phenotype, whereas IL-4 and IL-13 lead to polarization towards an M2-like phenotype [132, 133]. However, not only cytokine environment is a key player for macrophage activation and polarization; direct T cell-macrophage interaction has also been shown to be important. This has also been previously demonstrated by our group in the context of cancer, by showing that *in vivo* cognate interaction between tumor-infiltrating macrophages and Th1-type CD4$^+$ T cells results in a shift in macrophage phenotype from an M2-like to an M1-like state [134-138]. Both signals that are required for proper macrophage activation can be delivered by activated Th1 cells: secretion of IFN-γ and binding of the costimulatory CD40 ligand (CD40L) to the CD40 receptor expressed on the macrophage [139, 140]. Upon binding of CD40L, several signal transduction pathways are activated resulting in expression of genes involved in cell survival, production of pro-inflammatory cytokines, nitric oxide (NO), expression of co-stimulatory molecules and cell adhesion molecules [141, 142]. Moreover, Heusinkveld et al. have shown that Th1 cells are able to switch M2-like macrophages towards an anti-tumor M1-like phenotype also in humans [72].

1.4.6 TAMs as targets for cancer treatment

The tumor microenvironment of solid cancers is complex, dynamic, and differs greatly between different cancer types, but also among patients bearing the same class of malignancies [143, 144]. The extent and qualitative features of immune cell infiltrated in the tumor changes with tumor stage. Similar to the rate of T cell infiltration, the number and phenotype of intratumoral macrophages are correlated with survival rate and response to therapy [59, 64-66, 143]. In extreme cases, macrophages can account for up to 50% of the total tumor mass [144]. Due to their known influence on tumor growth and progression, their general immunosuppressive properties, and the finding that increased numbers of TAMs is associated with poor survival, TAMs have received attention as a potential therapeutic target for the treatment of cancer [145]. Several strategies have been developed for the targeting of TAMs at different levels, including the blockage of monocyte recruitment to the tumor mass

with a CCL2 blocking agent [146], suppression of TAM polarization with CSF1/CSF1R inhibitors and induction of macrophage apoptosis [145], or their use as targets for delivery of desired anti-cancer drugs to hypoxic tumor areas with subsequent induction of cancer cell death [147]. Another strategy for using TAMs as targets is based on the potential of macrophages for functional re-polarization. Thus, re-programming of TAMs into a tumoricidal M1-like state using nanoparticles, monoclonal antibodies, low-dose irradiation and immunomodulating hormones like thymosin-α or by activated Th1 $CD4^+$ T cells might provide another strategy for cancer treatment [145, 148, 149]. As described above, our group has demonstrated that Th1 $CD4^+$ T cells are able to trigger re-polarization of M2-like TAMs towards an M1-like phenotype. By using an antibody against the CD40 receptor, thereby mimicking the T-cell mediated activation signal, Beatty et al. (2011) showed that tumor infiltrating macrophages could be activated into an M1-like anti-tumor phenotype and subsequently promote tumor reduction in a mouse model [71].

1.4.7 Macrophage-targeted CAR T therapy

In recent work from our group it was shown that indirect immune responses triggered by $CD4^+$ T-cell-mediated re-polarization of TAMs was sufficient to eliminate disseminated cancer of the bone marrow (multiple myeloma) [138]. This mechanism of tumor killing is attractive, since it does not require direct recognition of the tumor cells by the T cell. It is therefore conceivable that this strategy might be less prone to processes of tumor escape, such as acquisition of new mutations resulting in the loss of tumor antigens. However, direct T cell-macrophage interaction within the tumor is crucial for ensuring TAM-mediated anti-tumor responses [150]. For this sort of indirect immunotherapy to be feasible in a patient setting, it is therefore critical to develop a method that allows efficient and selective activation and re-polarization of the TAM. Based on these observations, we hypothesized that a novel, universal approach for re-programming of TAMs might be attained through the use of CAR T cells recognizing surface antigens specifically expressed by TAMs. This hypothesis addresses the immunomodulatory effects of the CAR T cells rather than their direct cytotoxic effector functions. Previous findings have indicated that immune cells, including macrophages, that are in close proximity to activated CAR T cells might be influenced by granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFN-γ secreted by these T cells [151]. Targeting a TAM surface antigen bypasses the need for the presence of a particular tumor-specific antigen on the cancer cells,

and would conceivably be applicable to any type of cancer with a sufficient degree of macrophage infiltration. The utility of such interventions is likely to be dictated by the abundance of the TAM-specific target antigen and the ability of TAM-targeted CAR T cells to elicit the intended macrophage re-polarization with subsequent immune-stimulatory changes to the tumor microenvironment. Like conventional CARs targeting tumor specific antigens expressed on the tumor cell surface, identification of an appropriate target antigen on the TAM cell surface is required. In order to avoid a systemic activation of macrophages or killing of cells expressing the antigen the CAR T cell is specific for, it is critically important to identify antigens that are exclusively expressed or highly overexpressed on the surface of TAMs. A transient depletion of monocytes/macrophages might be acceptable, but a long-term suppression of these key players of the immune system can be highly problematic, and would impose a great risk of fatal infectious complications. Similarly, an unspecific activation of monocytes/macrophages might trigger systemic inflammatory responses resembling those observed in CRS, and possibly promote autoimmunity. Defining an appropriate target structure with limited expression in healthy tissue immune cells is therefore critical.

Several surface antigens have been reported to be selectively expressed by TAMs. This growing list includes the asparaginyl endopeptidase enzyme legumain, the folate receptor beta (FRβ) and the pattern recognition scavenger receptor MARCO [152-155]. At present, for the purpose of exploring the conceptual feasibility of a TAM-targeted CAR T, folate receptor beta (FRβ) was identified as a promising candidate [154, 155]. In mice, expression of FRβ is not observed in monocytes, and minimal expression is seen in healthy tissue macrophage subsets [156]. FRβ has been explored as a target of antibody-based therapies, showing impaired tumor growth [156-158].

1.4.8 Tumor cell death mediated by macrophages

Evading growth suppressors, resisting cell death and sustaining proliferation are only some of the hallmarks of cancer cells which enable them to proliferate in an uncontrolled manner. Furthermore, tumor cells have developed several strategies to evade the host's immune system. They do so by, amongst others, down-regulation of MHC class I expression, expression of proteins on their surface that silence effector functions of immune cells and secretion of immunosuppressive molecules and cytokines [159].

Understanding the mechanism underlying tumor cell death, is essential for the development of treatment methods. In normal cells, cell death can result from cellular/systemic stress, cellular injury, cellular signaling, inflammations and infections. In cancer cells, induction of tumor cell death can be accomplished with various methods, such as induction of autophagy, programmed necrosis, mitotic catastrophe and as in the case of most treatment methods induction of apoptosis [159].

Apoptosis, first described in 1972 [160], is a controlled cell death, which does not result in secretion of pro-inflammatory molecules. Once the process is activated, the cell begins to shrink and the membrane forms blebs. The cytoplasm and nucleus are enzymatically broken down and degraded cell components are enclosed in a membrane and subsequently phagocytized and degraded by macrophages [160]. Different factors can lead to induction of apoptosis. The "intrinsic" pathway is induced by DNA damage, withdrawal of growth factors/hormones, osmotic stress, toxins, viral infections and free radicals. The extrinsic" pathway is induced upon binding of e.g. TNF-α, Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) to receptors of the TNF receptor family expressed on the target cell. Both pathways lead to activation of the executioner caspases that degrade proteins by cleaving peptide bonds [159, 161, 162].

Macrophages have been shown to kill cancer cells by various mechanisms that will either lead to phagocytosis of the cancer cell or secretion of pro-inflammatory cytokines or cytotoxic mediators (e.g. oxygen radicals) which induces apoptosis or recruitment of other immune cells that then lyse the cancer cell. Phagocytosis of cancer cells can be induced after binding of a macrophage to an antibody-coated cancer cell, a mechanism referred to as antibody-dependent cellular cytotoxicity (direct killing) [1]. Furthermore, upon activation of a macrophage, either by IFN-γ and LPS or by binding to a Th1 cell (e.g. CAR T cell), production of inducible nitric oxide synthase (iNOS) is observed leading to the generation of nitric oxide radicals (·NO). ·NO itself is not very reactive but can diffuse across cell membranes and become very potent when it reacts with $O_2^{-}\cdot$ (a superoxide radical) to generate peroxynitrite ($ONOO^-$). $ONOO^-$ is a very toxic oxidant that through various mechanisms can causes damage to enzymes, DNA, membranes, and also triggers mitochondria to release apoptosis-inducing factor (AIF) and cytochrome *c*, resulting in the induction of apoptosis [163]. Our group has previously shown that generation of $ONOO^-$ happens within the cancer cell during killing, and that macrophage NO-induced killing of cancer cells is spatially restricted [164]. Hence,

repolarization of TAMs towards the M1-like phenotype (which results in production of iNOS and secretion of ·NO) using FRβ CAR T cells, can be a promising treatment method to kill tumor cells.

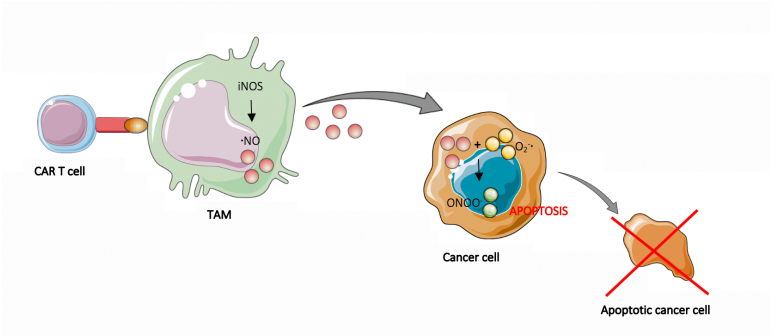

Figure 6. **Simplified illustration of macrophage NO-induced killing of cancer cells.** Upon activation of a TAM by a TAM-specific CAR T cell will result in, among others, expression of iNOS leading to generation of ·NO. ·NO is capable of diffusing across cell membranes, and reacts with $O_2^{-}\cdot$ to generate $ONOO^-$. $ONOO^-$ is a very toxic oxidant that through a number of mechanisms can induce apoptosis. CAR T cell, chimeric antigen receptor T cell; iNOS, inducible nitric oxide synthase; ·NO, nitric oxide; TAM, tumor associated macrophage; $O_2^{-}\cdot$, superoxide; $ONOO^-$, peroxynitrite. (Illustration was made by using components from Servier Medical ART.)

1.4.9 Interleukin-15 (IL-15)

In 1994, IL-15 was co-discovered by two different research groups [165, 166], and was characterized as a T cell growth factor. In the following years, more effector functions have been discovered, thus making it an important immunoregulator for both the innate and adaptive immune response. It also functions in non-hematological cells. IL-15 belongs to the four-α-helix bundle family and its mRNA is produced by various cell types such as DCs, macrophages and T- and B lymphocytes and non-myeloid cells (e.g. nerve cells, epithelial cells), whereas the protein form of IL-15 is limited to macrophages and DCs. Besides other functions, it regulates the activation and proliferation of NK cells, B and T lymphocytes, induces immunoglobulin synthesis by B cells and enhances maturation of DCs and phagocytosis activity of macrophages. Moreover, the production and secretion of pro-inflammatory cytokines like TNF-α and IFN-γ by macrophages and DCs, respectively, is induced by this potent cytokine. Consequently, expression and secretion of IL-15 must be highly regulated and limited to only a few cell types. The strategy used to prevent an uncontrolled expression of IL-15 in the periphery, is its trans-presentation on the surface of macrophages and DCs by the help of the receptor IL-15Rα. The bound IL-15 is then recognized by IL-2R/IL-15Rb and γc receptor subunits on for example T cells and NK cells [167]. Because of its broad activity pattern, an increased IL-15 expression level can have severe health effects

like development of different types of leukemia (a hematological malignancy), inflammation and autoimmune disorders (e.g. celiac disease) [167-170]. In contrast, preclinical studies in mice have shown that IL-15 plays a protective role against growth of solid tumors that do not arise from cells of blood-forming tissue. Several preclinical studies in which IL-15 was administrated alone or combined with the administration of a mAbs or other cytokines have been performed. Results seen in these studies indicate that IL-15 represents a promising candidate for cancer therapy [167].

1.5 CRISPR/Cas9 – A scalpel for the genome

The method is based on a natural (adaptive) defense system used by prokaryotes to protect against incorporation of exogenous DNA, e.g. through viral infection. This mechanism confers the ability to "remember" the virus and thus develop immunity.

Upon introduction of viral DNA into the bacterium, the DNA becomes integrated into the genome of the bacteria as a natural step during viral replication. A bacterial endonuclease cuts out a piece of the viral DNA and re-inserts it into the clustered regulatory interspaced palindromic repeats (CRISPR)-array of its genome, which in turn is transcribed into an RNA molecule. This CRISPR-RNA (crRNA) contains both information of the bacterium (e.g. *cas* genes) and the virus. crRNA will then bind to the partly complementary trans-acting CRISPR RNA (tracrRNA) and form an RNA double-helix, which will then bind to the Cas9 enzyme. Upon infection of the same type of virus, the Cas9 enzyme with its specific crRNA molecule recognizes and binds to the complementary viral DNA and cuts the strands, thereby making the virus harmless [171, 172].

Discovery of this naturally occurring (defense-) mechanism changed the way of working in the field of gene technology completely. Development of a modified form of the Cas9 protein makes it possible to apply this technique in cells with a nucleus.

With CRISPR/Cas9 it is possible to efficiently introduce site-specific modifications in the genome of many different organisms: disruption of a gene by introducing (targeted) deletions and insertions; introduction of a new gene (eventual novel gene); correction of a defective gene by replacing it with the correct sequence. Furthermore, by introducing mutations in the Cas9's activation domain, CRISPR/Cas9 can activate/down-regulate gene transcription upon fusion of dCas9 (rather than cleaving) to activator- or repressor domains. It also can be used for visualizing specific genomic loci [171, 173-175].

1.6 Aims of the study

Tumor associated macrophages (TAMs) can make up to 50% of the tumor environment and show an upregulated expression of the FRβ, hence making them theoretically capable of serving as targets for FRβ-specific CAR T cells. The aim of this master thesis was to investigate the effects of FRβ specific CAR T cells on tumor-infiltrating macrophages, and to evaluate their utility in the treatment of established solid tumors.

1. Generate and verify the functionality of FRβ CAR T cells.
2. Examine the ability of FRβ CAR T cells to recognize and repolarize TAM-like macrophages.
3. Evaluate the therapeutic efficacy of FRβ-specific CAR T cells in immunotherapy against advanced-stage solid tumors in immunocompetent mouse models.

2 METHODOLOGY

2.1 Mice

Wild type Balb/cAnNRj and C57BL/6NRj mice were purchased from the Janvier Labs, and Balb/c Rag1$^{-/-}$ from Taconic Bioscience. All animals were kept under special pathogen-free (SPF) conditions. All animal experiments were previously approved by the Norwegian Animal Research Authority (Mattilsynet; approval no. 17/179845-1) and performed in compliance with Federation of European Laboratory Animal Science Associations (FELASA) guidelines.

2.2 Cell Cultures

Thermo Scientific™ Nunc™ Cell Culture Treated EasYFlasks™, providing a hydrophilic surface allowing for an enhanced attachment of a variety adherent cell types to the flask surface and their growth, were used for culturing of all cell lines. Cells were cultured in their appropriate culture medium in a Heracell CO_2 150i incubator at 37˚C and in a humidified atmosphere with 5% CO_2, and routinely checked in the microscope. When reaching 80-90% confluency, cells were split. It is important to check each cell line's demands for nutrients, hormones and cytokines in order to allow proper cell growth, survival and differentiation state.

HEK293T cells were cultured in complete Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS, Gibco), 5 ml non-essential amino acids (100x MEM NEAA, Gibco), 1 mM sodium pyruvate (100 mM, Gibco) and 5mL 10.000 U/mL penicillin/streptomycin (100x, Gibco). All other cell lines were cultured in RPMI-1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated Fetal Bovine Serum (Gibco), 5 mL non-essential amino-acids (100 x MEM NEAA, Gibco), 1 mM sodium pyruvate (100 mM, Gibco) and 5 mL 10.000 U/mL penicillin/streptomycin (100x Pen Strep, Gibco).

2.3 Transfection of HEK293 cells

For transfection, cells with a low passage number should be in a logarithmic growth phase (<90% confluent) in order to achieve a good transfection efficiency. 10×10^6 cells/175cm^2 flask were sub-cultured for 24 hours prior to transfection start to ensure that cells were sufficiently recovered. 30 µg expression vector DNA (pMSCV-CL10-m28z-IRES-GFP or pMSCV-M315-m28z-P2A-mIL1RA), 12 µg CMV-driven *gag-pol* expression plasmid (pHIT60), and 6 µg CMV-driven ecotropic *env* expression plasmid (pHIT123) were incubated with 200 µL 25-kdL polyethylimine (PEI; 1 mg/mL, pH 7.0) in 3 mL OptiMEM® (Gibco) for 7 minutes, followed by

drop-wise addition of the DNA-PEI complex to the HEK293T cells. On the following day, medium was replaced with 30 mL OptiMEM® supplemented with 240µL of 0.5 M Valproic acid (VPA, Sigma-Aldrich). Retrovirus-containing supernatant was collected 24- and 48-hours following transfection, and up-concentrated by ultracentrifugation for 1.5 hours at 4 ˚C and 35.000 rpm. The expression vector used was constructed using the MSCV IRES GFP retroviral expression vector obtained from Dr. T Reja, Stanford University, through the Addgene repository (Addgene; #20672). Packaging vectors were generously provided by dr. R. Debets, Erasmus University Rotterdam.

2.4 Splenocyte collection and T cell culture

Spleen from wild type Balb/c or C57BL/6NRj mice was dissected out. To obtain a single-cell suspension, the organ was passed through a 70µm Cell Strainer (VWR, Cat no. 732-2758). Erythrocytes were depleted by incubation of the cell suspension in hypotonic lysis buffer I (17 mM Tris, 0.14 M NH_4CL, pH 7.2) for 5 minutes on ice, followed by washing with Phosphate Buffered Saline (PBS, Sigma) twice. Naïve $CD4^+$ lymphocytes were isolated by positive selection with magnetically labeled MicroBeads (CD4 (L3T4) MicroBeads, mouse, Miltenyi Biotec, Cat no. 130-117-043) using a LS MACS® Column (Miltenyi Biotec) and a MACS® Manual Separator for magnetic cell isolation (Miltenyi Biotec). For activation, $CD4^+$ T cells were cultured on a plate pre-coated with 0.5 µg/mL anti CD3 (BioXCell, clone 145-2c11, Cat no. BE0001-1) and 5 µg/mL anti-CD28 (BioXCell, clone 37.51, Cat no. BE0015-1) in complete RPMI supplemented with 50 µM 2-Mercaptoethanol (Sigma Aldrich, Cat no. M7522) and 1% Insulin-Transferrin-Selenium (ITS, Gibco). For an enhanced proliferation rate, 0.5 mg/mL murine IL-7 (Peprotech, Cat no. 217-17) and 30 U/mL murine IL-2 (Peprotech, Cat no. 212-12) were added on the following day, and cells were expanded for another 24 hours before they were used for retroviral transduction.

2.5 Retroviral Transduction of murine primary T cells

Two days prior to the transduction start, murine $CD4^+$ T cells were isolated from spleen and activated as previously described in section 2.4. On the day of transduction, retroviral supernatant (fresh or thawed) was diluted with two parts of OptiMEM® and transferred into a non-tissue culture treated 24-well plate (Sigma, Cat no. CLS3738-100EA) coated with 11 µg/mL RetroNectin® (TaKaRa/Clontech, Cat no. T100B-TAK). By centrifugation for 2 hours at

32˚C and 2000*g*, the virus particles were bound to the RetroNectin reagent. Then, 1 x 10^6 of activated $CD4^+$ T cells were resuspended in very little volume and added to the plate, followed by centrifugation for 30 minutes at 37˚C and at 800*g* and incubated over night at 37 ˚C and 5% CO_2. The next day, transduced cells were collected from the plate and expanded in complete RPMI medium containing 50 µM 2-Mercaptoethanol, 1% Insulin-Transferrin-Selenium (ITS, Gibco) and 30 U/mL murine IL-2. Transduction efficiency was assessed using flow cytometry two days post-transduction.

2.6 Macrophages (Mϕ)

In order to test whether CAR T cells are able to activate and re-polarize TAMs, murine bone marrow derived macrophages are used to evaluate the anti-tumor effect of the CAR T cell.

For this, bone marrow was dissected out from Balb/c mouse (older than 12 weeks), bone marrow cells isolated, erythrocytes depleted with hypotonic lysis buffer I for 5 minutes on ice, and in complete RPMI medium containing 20 ng/mL murine macrophage-colony stimulating factor (mM-CSF, Peprotech, Cat no. 315-02) differentiated into macrophages. Fresh complete RPMI medium with 20 ng/mL mM-CSF was added to the cell culture on day+3. On day+6 the uncommitted macrophages (M0-like phenotype) were ready for polarization.

For M1-like phenotype polarization, macrophages were primed with 150 U/mL recombinant mouse IFN-γ (Peprotech, Cat no. 315-05) for 4 hours, followed by stimulation with 100 ng/mL Escherichia coli O127:B8 lipopolysaccharide (LPS, Sigma-Aldrich, Cat no. L3129) for 20 hours.

M2-like phenotype polarization was induced by incubation with 20 ng/mL murine IL-4 for 48 hours (Peprotech, Cat no. 214-14). TAMs were generated by co-incubation of M0-like macrophages with tumor cell-conditioned medium (TCM), preferably a murine melanoma cell line (B16), for 48 hours.

Activation state of the macrophages was evaluated by expression of certain surface markers using flow cytometry. For M1-like macrophages CD86 and MHC-II were used and for M2-like macrophages CD206 (mannose receptor) and FRβ.

2.7 Flow cytometry analysis

Antibodies used for characterization of macrophages and evaluation of transduction efficiency of retroviral transduction of murine $CD4^+$ lymphocytes are listed in **Table 1**.

Non-specific binding was blocked by incubation of cells in PBS containing 1% bovine serum albumin (BSA, Bio-Rad, Cat no. 805095) and 50 µg/mL anti-FcγRII/III monoclonal antibody (HB-197^{TM}, ATCC, clone 2.4G2) on ice for 30 minutes. Cells were stained with specific antibodies in PBS containing 0.5% BSA for 30 minutes on ice and in darkness. Cells were analyzed by flow cytometry (Fortessa, BD, Pharmingen) and data processed using FlowJo 10.4 Analysis Software (v3.05478).

Table 1: **Antibodies used for flow cytometry analysis.**

Specificity	Clone	Conjugation	Isotype	Used conc.	Company	Cat no.
CD11b	M1/70	FITC	Rat IgG2b	1:60	SB	1561-02
CD206	C068C2	PE	Rat IgG2a, κ	1:100	BL	141706
MHC-II I-A/I-E	M5/114.15.2	PacBlue	Rat IgG2b, κ		BL	107620
CD86	GL1	APC	Rat IgG2aκ	1:200	SB	1735-11
FOLR2 (FRβ)	Polyclonal	Unconjugated	Rat IgG	1:50	Abgent	AP5032a
Id-315	-	Unconjugated	Mouse IgG1	1:100	Homemade	-
FRβ	-	Biotin	scFv	2 µg/mL	Homemade	-
Rabbit IgG	-	A488	Goat IgG	2 µg/mL	TMO	A11008
Rabbit IgG	-	A647	Goat IgG	2 µg/mL	TMO	A21244
Mouse IgG	-	A647	Goat IgG	2 µg/mL	TMO	A32728
Streptavidin	-	APC/Cy7	-	1:200	BL	405208

BL = BioLegend, SB = Southern Biotech, MP = Molecular Probes, LT = Life Technologies, TMO = ThermoFisherScientific

2.8 *In vitro* Assays

2.8.1 Macrophage cytotoxicity assay (JAM Assay)

JAM assay is a method to determine the amount of cell death by measuring DNA fragmentation. Amount of DNA retained in living cells is measured rather than the release of radioactivity from the dying cells. For this, actively dividing target cells are radioactively labeled by incorporation of [^{3}H]-labeled nucleotides into their DNA. Upon cell death, DNA fragmentation occurs. When the cells are harvested onto a fiberglass filter, only large pieces of DNA are captured onto the filter. Smaller DNA fragments of dead cells will pass through the filter, thus only DNA from viable cells is quantified by counting the radioactivity of the bond [^{3}H]. For macrophage cytotoxicity assay, bone marrow derived cells were isolated and differentiated into macrophages as previously described in section 2.6. Differentiated macrophages were seeded in flat-bottom 96-well plates (0.12×10^6 cells/well) and allowed to

attach to the bottom of the wells before further use. Polarization towards an M2-like and TAM-like phenotype was obtained by a 48 hours incubation with 20 U/mL of murine recombinant IL-4 or supernatant of a B16 cell line, respectively. For activation and re-polarization of M2-like and TAM-like phenotype towards the M1-like state, macrophages were co-cultured with 0.5×10^5 CAR T cells for 24 hours. Mouse myeloma cells (MOPC315.4) were radioactively labeled by incubation with 5µCi/mL [methyl-^{3}H] thymidine (^{3}H-TdR, Montebello Diagnostics) for 6 hours prior to transfer to the macrophage-CAR T cell co-culture. After 18 hours, cells were harvested with a 96-well plate cell harvester (Microbeta Filtermat-96 Cell Harvester, PerkinElmer®, Part Number D961962) onto a glassfibre filtermat (PerkinElmer, cat no. 1450-421), dried in a microwave for 4 minutes at 700W and put in an appropriate plastic bag (PerkinElmer, cat no. 1450-432). 4mL of biodegradable liquid scintillation fluid (High Flash-point LSC cocktail, PerkinElmer®, Cat.no 1205-440) was added to wet the filter and radioactivity was determined in a beta counter (1450 MicroBeta TriLux). Percentage of killing, determined by ^{3}H-TdR release in counts per minutes (cpm), was calculated as follows:

$$\textbf{\% DNA fragmentation = \% killing} = 100 \times \frac{\text{cpm tumor cells cultured alone} - \text{cpm tumor cells cultured with M}\phi\text{s}}{\text{cpm tumor cells cultured alone}}$$

Polarization of M0-like Mϕs towards the M1-like state 24 hours prior to transfer of [^{3}H]-thymidine labeled MOPC-315.4 cells to the wells, was used as a positive control.

As a negative control, unstimulated M0-like Mϕs were co-incubated with [^{3}H]-thymidine labeled MOPC-315.4.

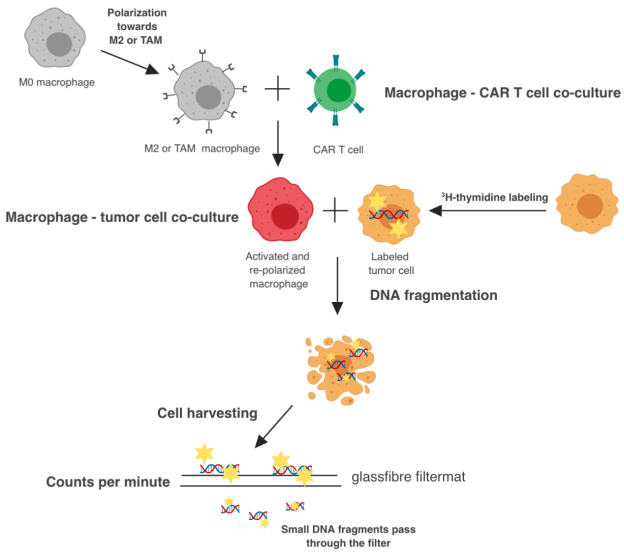

Figure 7. **Workflow of macrophage cytotoxicity assay (JAM Assay).** (Illustration was made by using components from BioRender.)

2.8.2 Griess nitrite assay

Macrophages in their pro-inflammatory state produce inducible nitric oxide synthase (iNOS) leading to the generation nitric oxide radicals (·NO). ·NO is capable of diffusing across cell membranes and can generate the more potent peroxynitrite ($ONOO^-$) in presence of superoxide radicals ($O_2^{-}\cdot$). $ONOO^-$ is a very toxic oxidant that through a number of mechanisms can induce cell death.

The Griess reaction, first described by Griess [176], measures spectrophotometrically the amount of nitrite anion (NO_2^-; a stable breakdown product of NO), which is used as an indicator of NO production. Under acidic condition, NO_2^- is reduced to nitrous acid (HNO_2) that reacts with sulphanilamide to form an intermediate compound to which naphthylethylene diamine is coupled. This results in formation of a purple azo compound that absorbs light between 520 and 550 nm.

For Griess nitrite assay, differentiated macrophages were seeded in flat-bottom 96-well plates (0.12×10^6 cells/well) and allowed to attach to the bottom of the wells before further use. Polarization towards a TAM-like phenotype was obtained by a 48 hours incubation with supernatant of a B16 cell line. For activation and re-polarization towards the M1-like state, TAM-like macrophages were co-cultured with 0.5×10^5 CAR T cells. After 24 hours, culture medium was collected from the appropriate wells. Activation state of re-polarized TAMs was evaluated by measuring NO production using the Griess Reagent System (Promega, Cat no. G2930). For this, 50 µL of culture media was first incubated with 50 µL of sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) for 10 minutes at room temperature (RT) and in darkness, before 50 µL of NED solution (0.1% N-1-napthylethylenediamine dihydrochloride in water) was added to the wells, followed by a 10 minutes incubation at RT and in darkness. Then, the absorbance was measured at 540 nm using a microplate reader (EnVision 2104 Multilabel Reader, PerkinElmer). Nitrite concentration was calculated based on a standard curve using 0.1M sodium nitrite ($NaNO_2$) dissolved in complete RPMI-1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated Fetal Bovine Serum (Gibco), 5 mL non-essential amino-acids (100 x MEM NEAA, Gibco), 1 mM sodium pyruvate (100 mM, Gibco) and 5 mL 10.000 U/mL penicillin/streptomycin (100x Pen Strep, Gibco).

2.8.3 Bio-Plex Multiplex Assay

The Bio-Plex Multiplex Assay is based on color-coded magnetic beads that are used for detection of multiple cytokines, growth factors, chemokines in tissue culture supernatants, plasma and serum. The use of color-coded beads makes it possible to quantify levels of multiple proteins in a single sample. The principle of this assay is similar to the sandwich immunoassay format. First, capture antibody-coupled beads are dispensed to each well of a 96-well microplate and co-incubated with the samples to allow binding of the beads to their targets. After unbound proteins are washed away, biotinylated detection antibodies are added to the wells resulting in formation of antibody-target complex. In the final step, streptavidin-phycoerythrin reporter dye (binding to the biotinylated detection antibodies) is added to the sample. Using a Bio-Plex Multiplex Reader, the fluorescence of the beads and of the bound streptavidin-phycoerythrin reporter dye is measured. The intensity of the fluorescent reporter signal indicates the relative quantity of target biomarkers in the sample.

For the Bio-Plex Cytokine Assay, supernatant from *in vitro* macrophage-CAR T cell co-cultures was collected and the concentration of multiple cytokines were measured using Bio-Plex Pro™ Mouse group I 23-Plex Panel Assay (Bio-Rad, Cat no. M60009RDPD) according to the manufacturer's provided protocol. Fluorescence was measured using a MAGPIX™ Multiplex Reader (Bio-Rad) and data was analyzed with Bio-Plex Manager™ software 6.0. The cytokines detected included IL-1β, IL-6, IL-12p70, IFN-γ and TNF-α.

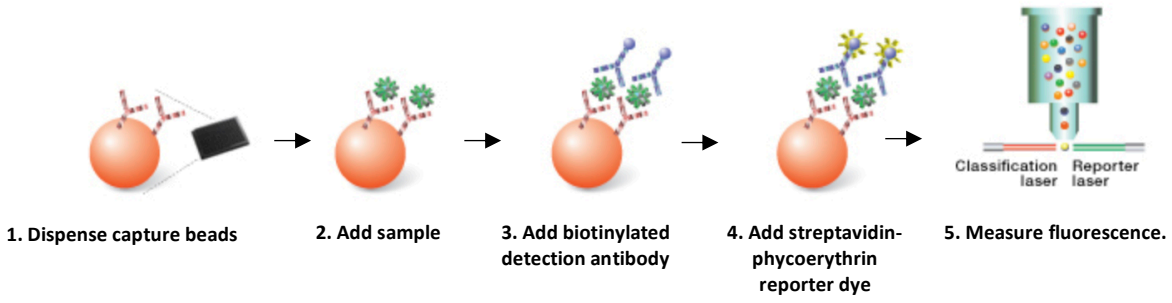

Figure 8. **Workflow of Bio-Plex Cytokine Assay.** (Adapted from Bio-Rad)

2.9 Generation of B16 FRβ knockout cell line by CRISPR/Cas9

Murine melanoma cell line (B16) deficient in FRβ expression was achieved by introduction of a non-sense mutation within the FOLR2 (FRβ) gene. The web-based target design tool Chop-Chop (http://chopchop.rc.fas.harvard.edu/) was used to design single guide RNA (sgRNA) sequences (**Table 2**) targeting sites within the FOLR2 gene (Gene ID: 14276), with no predicted off-target binding sites in the murine genome.

FOLR2_s1 and FOLR_as1 or FOLR2_s2 and FOLR_as2 were annealed with each other, followed by insertion into the pSpCas9(bb)-2A-GFP (PX458) vector, encoding a chimeric guide RNA (gRNA) plus eGFP and human codon-optimized Cas9. PX458 was a gift from Feng Zhang provided through the Addgene repository (Addgene plasmid # 481380). Next, 2×10^6 B16 cells, suspended in nucleofection buffer (5 mM KCl, 15 mM $MgCl_2$, 120 mM Na_2HPO_4/NaH_2PO_4; pH 7.2). 2.5 µg of PX458 was delivered into B16 cells by electroporation using an Amaxa Nucleofector (Lonza Inc., Basel, Switzerland). Plasmid uptake was confirmed by GFP expression in cells 24 hours later, and GFP-positive cells were sorted by single-cell sorting using a FACS Aria II (BD Bioscience). Sorted cells were expanded in complete RMPI medium. Genomic DNA was isolated from individual clones using a GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific, Cat no. K0721). Gene fragments spanning the intended sgRNA target sites were screened for non-sense mutations following polymerase chain reaction (PCR) amplification using the primer pairs listed in **Table 2**. To this end, PCR fragments were electrophoresed on 2.5% agarose gels, purified using QIAquick Gel Extraction Kit (Qiagen, Cat no. 28706) and individual fragments cloned using TOPO PCR Cloning (ThermoFisher Scientific, Cat No. 450245), followed by transformation into chemically competent TOP10 *Escherichia Coli* (*E. Coli)*. For each PCR product, 6-8 TOP10 transformants were expanded, plasmid DNA isolated by miniprep purification (Wizard®Plus SV Minipreps DNA Purification System, Promega, Cat no. A1460), and submitted for Sanger sequencing. Clones harboring two different frameshift mutations in all the sequenced colonies, resulting in biallelic ablation of the *FOLR2* (FRβ) gene, were selected for further culturing.

Table 2: **Sequences of designed sgRNAs and primers used for PCR.**

Name of sgRNA	Sequence (5'-3')	Seq. Primer (5'-3')	Tm (˚C) (Phusion)	Exp. Length (bp)	Tm (˚C) optimal
FOLR2_s1	GGTCACAACAGGCAGTGGCC	**FOLR2_1_fw** GGTCTCCCTAACTTTAGCTCCC	61.2	260	64.7
FOLR2_as1	GGCCACTGCCTGTTGTGACC	**FOLR2_1_rev** GTGTGTCTGGCTGCTCACTAAC	62.7		
FOLR2_s2	GTGAGTAGGTCTGTTCTGTCC	**FOLR2_2_fw** CTGGAAACGACACCACTCTTG	60,4	259	64.0
FOLR2_as2	GGACAGAACAGACCTACTCAC	**FOLR2_2_rev** TACTTTCCACCAAGCCATCTCT	60.9		

2.10 *In vivo* tumor challenge and adoptive cell transfer (ACT) of CAR T cells

Adult mice C57BL/6NRj (older than 12 weeks) were injected with cancer cells suspended in 100 µL PBS. Tumor development and health status of the animals was monitored daily by palpation and tumor size measured with a caliper. Mice were euthanized by cervical dislocation when the tumor size reached 20 mm, the animals lost ≥20% of their initial weight, or showed any other sign of serious illness.

For adoptive cell transfer experiments, mice were challenged with B16 cells, Lewis lung carcinoma (LL/20) cells, MOPC315.4 cells or murine colon carcinoma (CT26) cells (**Table 3**). When tumor size reached five mm in diameter, mice were irradiated with 4 Gy using an Xstrahl RS320 X-Ray irradiator (Xstrahl Ltd, Surrey, England). One day later, 1×10^6 specific CAR $CD4^+$ T cells (suspended in 100 µL PBS) were injected intravenously (i.v.) in the lateral tail vein. As a negative control, M315 specific CAR $CD4^+$ T cells were used. M315 is a monoclonal IgA myeloma protein secreted by murine MOPC315 myeloma cells. Prior to ACT, $CD4^+$ T cells were isolated from spleens of wild type Balb/c or C57BL/6NRj mice, activated and expanded in presence of 30 U/mL of murine IL-2 (Peprotech, Cat no. 212-12) for two days, followed by retroviral transduction with the corresponding CAR construct. After ACT of CAR $CD4^+$ T cells, tumor volume was estimated by measuring tumor width (W), tumor depth (D) and tumor length (L) with a caliper every second day, and calculated using the following formula:

$V = \pi/6 \times L \times W \times D$. Sick animals were treated with 0.5 to 1 mL Ringer's acetate solution (subcutaneous injections).

Table 3: **Cancer cell lines used for *in vivo* administration.**

Cancer cell line	Mouse Model	Number of cells per 100 µL PBS	Injection volume	Form of injection
CT26	Balb/c	3×10^5	100 µL	s.c.
LL/2	C57BL/6NRj	3×10^5	100 µL	i.d.
B16	C57BL/6NRj	3×10^5	100 µL	i.d.
MOPC315.4	Balb/c	3×10^5	100 µL	s.c
CT26	Balb/c Rag1 $^{-/-}$	3×10^5	100 µL	s.c.

s.c.= subcutaneous, i.d. = intradermal, CT26 = murine colon carcinoma cells, LL/2 = Lewis lung carcinoma, MOPC315.4 = mouse myeloma cells, B16 = murine melanoma

2.11 Statistical analysis

For tumor challenge experiments, survival curves were analyzed with the log-rank test. Mann-Whitney U test were used to calculate p-values. Statistical analysis was performed with GraphPad Prism 7. $p < 0.05$ was considered to be statistically significant.

3 RESULTS

3.1 Generation of FRβ-specific CAR T cells

Adoptive transfer of lymphocytes is an evolving strategy to redirect T cells towards a specific antigen, such as tumor antigens. In the past few years, CD19-spefic CAR T cell therapy has shown impressive results in the treatment of B-lymphoid malignancies [116-120].

However, solid tumors most often do not express nor secret tumor specific antigens. TAMs with their regulatory influence on tumor growth and progression [145], especially in already established tumor masses, have received increasing attention as potential therapeutic target. Previously published data has revealed that TAMs [153-155] and cancer types including acute myeloid leukemia (AML), lung adenocarcinoma and pancreatic cancer [154, 155, 158, 177] show an up-regulated expression level of folate receptor beta (FRβ). To this end, advantage was taken of a previously published scFv sequence derived from the rat anti-mouse anti-FRβ antibody CL10 [178]. Sequences from two mouse anti-human FRβ antibodies were also available (m909 and m923) [179].

Using *de novo* gene synthesis through a commercial service provider (Genscript), a 2nd generation CAR construct was generated comprising an IL-2 signal peptide (to promote entry of the fusion protein into the endoplasmic reticulum (ER)), the CL10 scFv coding region, sequences encoding the murine CD28 transmembrane and signaling domains, and the murine CD3 zeta (CD3ζ) domain (pMSCV CL10-m28z IRES EffLuc). A human equivalent was also generated, comprising the human homologue of the CD28 and CD3z domains. The constructs were generated in a modular fashion with restriction enzyme sites allowing swapping of scFv fragments. The CAR encoding constructs were then cloned into the multiple cloning site (MCS) of a bicistronic pMSCV retroviral vector, also containing an internal ribosomal entry site (IRES), followed by the coding sequence of enhanced firefly luciferase (EffLuc) [180]. The EffLuc tag allows bioluminescence tracking of cells upon adoptive transfer.

As negative controls, retroviral vectors encoding a CAR of irrelevant specificities (pMSCV M315-m28z IRES EffLuc and pMSCV NIP-m28z IRES EffLuc), and a vector with green fluorescent protein (GFP) in place of the CAR (pMSCV GFP IRES EffLuc) we generated. To functionally verify the constructs, our group established a staining protocol using his-tagged recombinant murine FRβ extracellular fragment (rmFOLR2) as a bait to verify intact target binding. Following T-cell transduction is was confirmed that T cells transduced with FRβ CARs

bound specifically to rmFOLR2, compared to irrelevant controls (hapten 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) CAR and M315 CAR) (*data not shown*). Next, MOPC315.36 myeloma cells were utilized that ectopically express surface-bound murine FRβ to test for T cell activation and target killing upon recognition by the CAR. FRβ CARs efficiently and selectively killed murine FRβ-expressing MOPC315.36 cells, and strong induction of IL-2 was observed in FRβ CAR T cells incubated in the presence of target-expressing cells (*data not shown*). Hence, it was concluded that our FRβ specific CAR is functional, and can be efficiently expressed in primary murine T cells.

3.2 FRβ-specific CAR T cells activate and polarize TAMs towards M1-like phenotype *in vitro*

Recent findings from our group, showing that, upon $CD4^+$ T cell engagement, macrophages are not killed, but re-polarized into a pro-inflammatory phenotype promoting the elimination of disseminated cancer of the bone marrow [138, 150]. We therefore hypothesized that targeting FRβ by the use of CAR T cells could be used to target TAMs with subsequent activation and re-polarization towards an M1-like anti-tumor phenotype.

We first assayed the effect of $CD4^+$ and $CD8^+$ FRβ CAR T cells on macrophages, to determine whether CAR engagement induced killing or functional re-polarization. Quantitation of living macrophages following 24-hour co-incubation with CAR T cells revealed a significant decrease in macrophage numbers following $CD8^+$ CAR T co-culture, whereas co-culturing with $CD4^+$ CAR T resulted in non-significant changes in macrophage numbers (**Fig. 9**). These results are in agreement with previous findings suggesting that $CD4^+$ T cell/macrophage interactions are non-cytotoxic [138, 150]. Given our aim of exploring the potential of T cell-mediated macrophage re-polarization, in this work we therefore opted to focus on $CD4^+$ CAR T cell preparations.

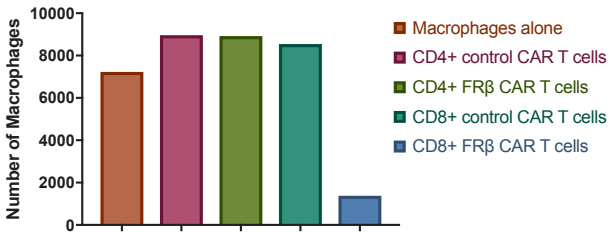

Figure 9. **Number of macrophages after co-culturing with CAR T cells for 24 hours.** Bone marrow derived cells were isolated and differentiated into macrophages in the presence of M-CSF. Polarization into M2-like states was initiated on day+6 with 20 ng/mL IL-4, followed by transfer of CAR T cells to the macrophage cultures on day+8. On the following day, number of living macrophages was quantified. Macrophages cultured in the absence of T cells were used as baseline.

For T cell-mediated macrophage polarization assays *in vitro*, it was important to verify the polarization and activation state of the macrophages before they were co-cultured with FRβ-specific CAR T cells. As further detailed in the materials & methods section (page 28), bone marrow derived cells were isolated and *in vitro* differentiated into macrophages under the influence of murine M-CSF. Polarization towards the TAM- and M2-like phenotype was induced with tumor cell-conditioned medium (TCM) and IL-4, respectively. The M1-like state was stimulated with IFN-γ and LPS. Unstimulated, M0-like, macrophages were cultured in medium without polarization factors and used as baseline. To verify the activation state, change in expression levels of CD86 and MHC class II (MHC-II); surface markers for M1-like

macrophage activation, and FRβ and mannose receptor (CD206); surface markers for M2 macrophages; were evaluated by flow cytometry. Compared to unstimulated M0 macrophages (red populations) and M1 macrophages (blue population), there was a shift in the histograms of IL-4 or TCM stimulated macrophages (orange and bown populations in **Fig. 10B** and red in **Fig. 10E**), indicating higher expression of M2 surface marker CD206 and FRβ (**Fig. 10B,E**). Expression of the M1 surface markers MHC-II and CD86 was similar or slightly decreased compared to M0, whereas M1 macrophages showed high expression (**Fig. 10C,D)**. We thus concluded that these *in vitro* differentiated and polarized macrophages were found to be in an M1-, M2- and TAM-like states at the onset of the experiment. Next, macrophage populations were co-cultured with CAR T cells for 24 hours. Prior to the transfer of T cells, transduction efficiency was checked by performing flow cytometry and numbers of T cells transferred to the macrophage cultures adjusted according to the transduction efficiency (**Appendix Figure A1**). To look for signs of macrophage re-polarization, we analyzed macrophage surface markers by flow cytometry, and cytokine production was quantified performing Bio-Plex multiplex immunoassays. To address whether antigen-mediated engagement of the CAR during T cell-macrophage interaction is crucial for ensuring TAM-mediated anti-tumor responses [150], macrophages were also co-incubated with control CAR T cells (M315 CAR T cells), which bind to an irrelevant antigen (trinitrophenyl hapten) not found on macrophages [181]. Compared to untreated M2 macrophages and TAMs (red populations), there was a shift in the histograms of macrophages co-cultured with FRβ specific CAR T cells (orange populations), indicating an up-regulation of M1 surface markers CD86 and MHC-II (**Fig. 10G,H,J,K**) and down-regulation of M2 surface marker CD206 (**Fig. 10F,I**). In contrast, the histograms in **Fig. 10F-K** showed no shifts when macrophages were co-cultured with control M315 CAR T cells (blue populations), indicating that these macrophages did not get re-polarized. Moreover, secretion levels of TNF-α, IFN-γ, IL-1β, IL-6 and IL12p70, cytokines normally secreted by pro-inflammatory macrophages, were increased in macrophages co-cultured with FRβ-specific CAR T cells compared to unstimulated TAM (**Fig. 11A-E**). As a positive control, cytokine production of *in vitro* polarized M1 state macrophages was measured. No up-regulated secretion of the mentioned cytokines was observed in macrophages co-cultured with M315 CAR T cells (**Fig. 11A-E)**, indicating that FRβ-specific CAR T cells, but not control CAR T cells, are able to bind to TAM- and M2-like macrophages with subsequent activation and polarization towards a pro-inflammatory state. These results show

that our FRβ-specific CAR T cells have the ability to activate and re-polarize TAMs and M2-like macrophages towards the M1-like phenotype.

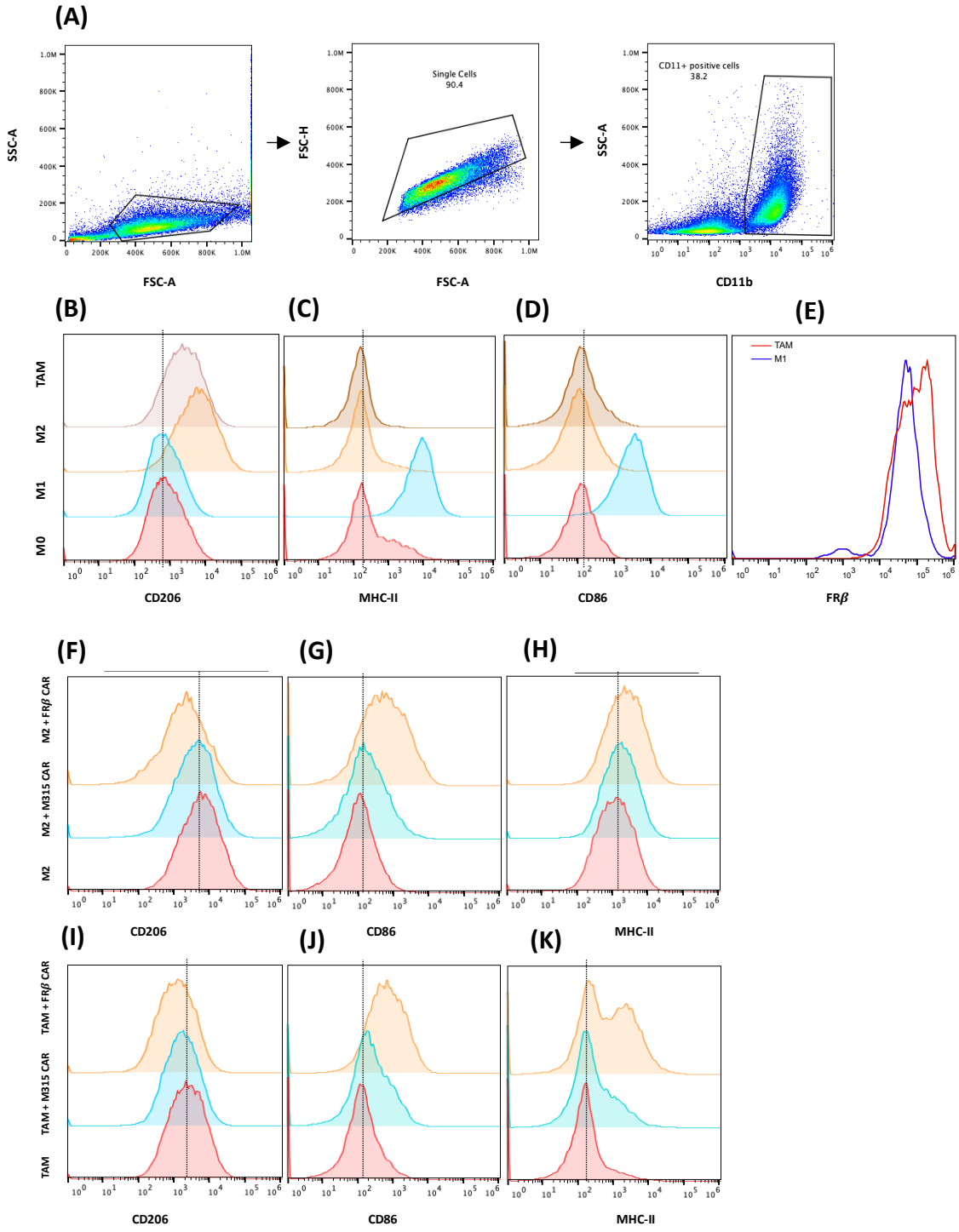

Figure 10. **Expression pattern of surface markers of *in vitro* differentiated and polarized macrophage subsets.** Bone marrow derived cells were isolated and differentiated into macrophages in the presence of M-CSF. Polarization into M2- and TAM-like states was initiated on day+6 with IL-4 and tumor cell-conditioned medium (TCM), respectively. Polarization towards the M1 state was initiated with IFN-γ and LPS on day+7. On day+8, FRβ-specific CAR T cells or control CAR T cells were added to M2- and TAM-like macrophage cultures. Activation state was analyzed by flow cytometry the next day. **(A)** Macrophage population was defined on forward and side scatter and single cells have been gated on CD11b. **(B-K)** Surface markers were examined on CD11b$^+$ gated cells. The figure shows data from one representative experiment conducted several times with similar results.

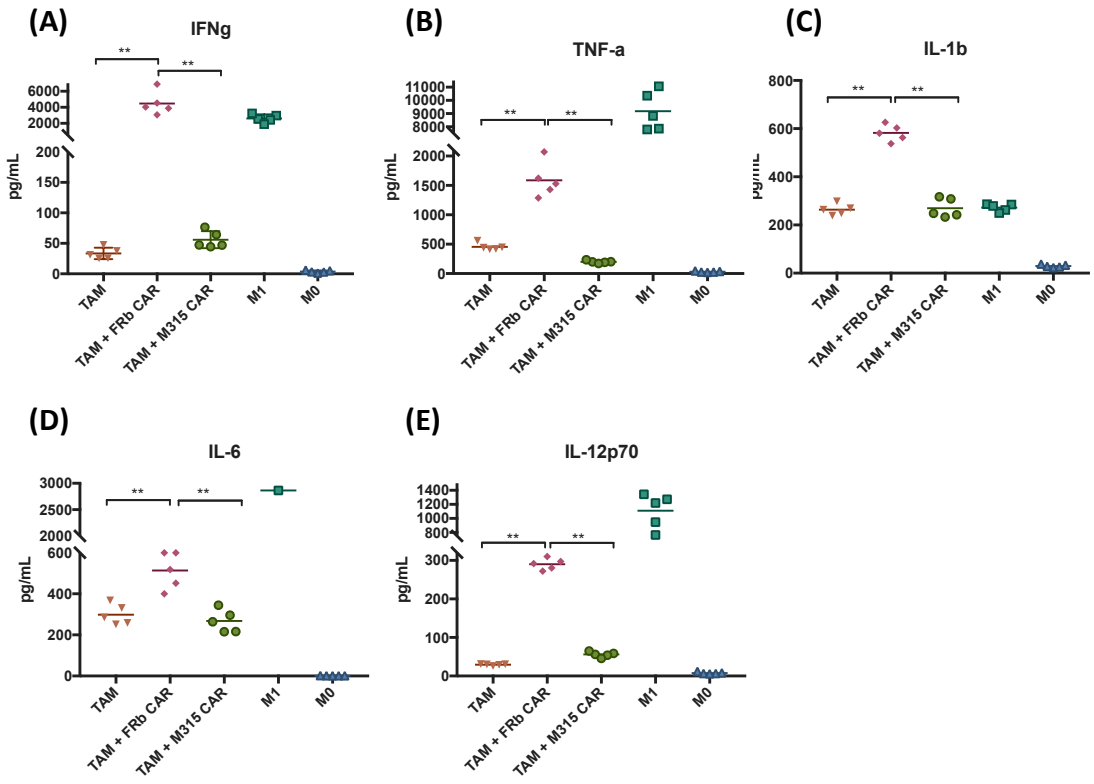

Figure 11. **Quantification of cytokine production of TAM- and M2-like macrophage subsets co-cultured with FRβ-specific CAR T cells or control CAR T cells.** Bone marrow derived cells were isolated and differentiated into macrophages in the presence of M-CSF. Polarization into the TAM-like state was initiated on day+6 with tumor cell-conditioned medium (TCM). Polarization towards the M1 state was initiated with IFN-γ and LPS on day+7. On day+8, FRβ-specific CAR T cells or control CAR T cells were added to the TAM-like macrophage cultures. Cytokine production was quantified performing Bio-Plex Multiplex Immunoassay on the following day. As positive control, secretion levels of classically activated (M1) macrophages was quantified. **(A-E)** Secretion levels of different cytokines were quantified. ** p-value<0.01

3.3 FRβ-specific CAR T cells have anti-tumor effect *in vitro*

We further investigated whether CAR-based activation and re-polarization of TAMs was sufficient to induce a tumoricidal macrophage phenotype. The ability of TAMs to kill mouse myeloma cells (MOPC315.4) was tested in an *in vitro* tumor killing assay (JAM assay). Bone marrow derived cells were isolated, differentiated into macrophages and polarized towards the TAM-like phenotype, followed by co-incubation with FRβ-specific CAR T cells. Prior to transfer of tumor cells to the macrophage-CAR T cell co-cultures, MOPC315.4 cells were radioactively labeled with [methyl-^{3}H] thymidine [182-186]. After 18 hours, cells were harvested, and percentage of killing was determined by measuring ^{3}H-TdR release in a beta counter. Tumor killing activity of TAMs co-cultured with FRβ-specific CAR T cells was compared to TAMs co-cultured with M315 CAR T cells and TAMs alone. As a positive control, IFN-γ/LPS was used to induce archetypical M1-state macrophages. JAM assay results revealed that TAMs co-cultured with FRβ-specific CAR T cells (red bar) were able to kill up to 84% of the MOPC315.4 cells (**Fig. 12A**), indicating that FRβ specific CAR T cells bound to and thereby activated TAMs towards a tumoricidal phenotype. Of note, tumor killing rate of macrophages re-polarized by FRβ specific CAR T cells was comparable to the killing rate caused by IFN-γ/LPS- activated M1 macrophages (blue bar). On the other hand, TAMs co-cultured with the control M315 CAR T cells (green bar) as well as TAMs and unstimulated M0 like macrophages alone with MOPC315.4 cells (orange and blue-green bars) did not show any anti-tumor effects (**Fig. 12A**). These results are in agreement with previous findings from our group, showing that T cell-macrophage interaction is crucial for TAM mediated elimination of tumor cells [150]. Furthermore, induction of iNOS production in activated tumoricidal macrophages in response to IFN-γ and LPS or upon interaction with Th1 cell is well described [163, 164]. To verify the role of iNOS and NO in macrophage mediated tumor killing, NO production was quantified using the colorimetric Griess assay. iNOS activity blocked by pre-treating the macrophage-T cell co-cultures with the selective iNOS inhibitor 1400W two hours prior to the addition of tumor cell. Resting M0, unstimulated TAMs and TAM-M315 CAR T cell co-cultures showed undetectable levels of NO secretion compared to TAMs co-cultured with FRβ specific CAR T cells (**Fig. 12C**). Furthermore, the presence of 1400W completely abrogated macrophage-mediated tumor killing (**Fig. 12B**), indicating that iNOS is responsible for NO secretion, and that NO is involved in macrophage mediated tumor killing *in vitro*. These results are in line with recent publications from our group, showing that iNOS-mediated macrophage killing is

responsible for tumor rejection by TAMs re-polarized by T cell receptor-transgenic CD4^{+} T cells [164].

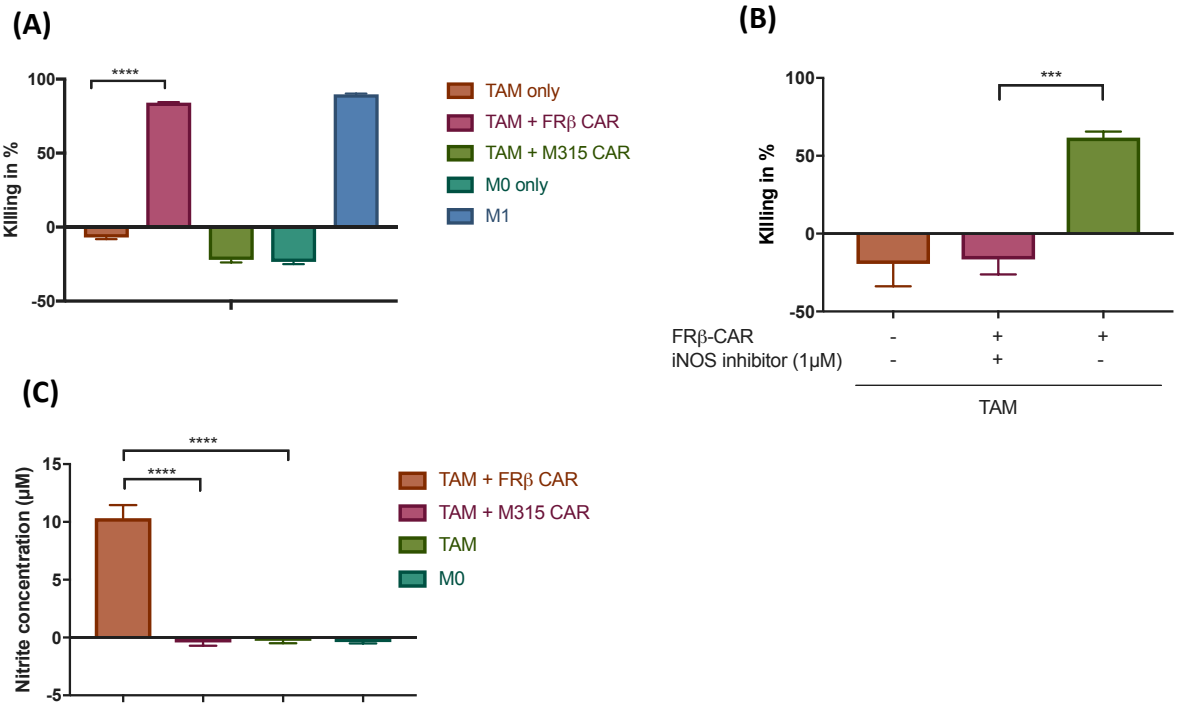

Figure 12. **Killing of mouse myeloma cells by activated and re-polarized TAMs *in vitro*. (A)** *In vitro* elimination of tumor cells was evaluated performing macrophage cytotoxicity assay (JAM assay). For this, bone marrow derived cells were isolated and differentiated into macrophages in the presence of M-CSF. Polarization into the TAM-like activation state was initiated on day+6 with tumor cell-conditioned medium (TCM). On day+8 FRβ-specific CAR T cells or control CAR T cells were added to the macrophages. TAMs co-incubated with FRβ-specific CAR T cells were re-polarized and killed up to 84% of the tumor cells, whereas TAMs co-cultured with control M315 CAR T cells showed no killing activity. Co-culturing of TAMs or unstimulated M0 like macrophages alone with MOPC315.4 cells did not cause tumor cell death. n=8 per treatment group and results are shown as mean + SD **(B)** Pre-treating the macrophage-T cell co-culture with the iNOS specific inhibitor 1400W two hours prior to the addition of [methyl-^{3}H] thymidine labeled tumor T cells led to complete abrogation of the macrophage-mediated cytotoxicity. n=8 per treatment group and results are shown as mean+SD **(C)** Griess assay showing the NO release from TAMs alone or co-cultured with FRβ-specific CAR T cells or control M315 CAR T cells. As positive control, IFN-γ/LPS stimulated macrophages were used. n=9 per treatment group and results are shown as mean+SD. *** p-value<0.001; **** p-value<0.0001

3.4 Adoptively transferred CAR T cells show anti-tumor effect *in vivo*

The findings from *in vitro* experiments demonstrated that FRβ-specific CAR T cells had the ability to activate and re-polarize TAMs towards a tumoricidal phenotype, with subsequent elimination of surrounding cancer cells, and secretion of pro-inflammatory cytokines. To evaluate the therapeutic potential of FRβ-specific CAR T cells in a more clinically relevant setting, we conducted adoptive cell transfer experiments in different mouse models. Balb/c, C57BL/6NRj and Balb/c Rag1$^{-/-}$ mice were challenged with various cancer cell lines and adoptively transferred with *in vitro* generated CAR T cells (1 x 10^6 cells per animal) when tumors reached a diameter of around five mm.

3.4.1 Adoptive transfer of FRβ-specific CAR T show anti-tumor effects against established murine melanoma (B16) *in vivo*

Adoptive transfer of FRβ CAR T cells resulted in significantly increased survival compared to control CAR T-treated mice, with complete tumor regression in three of eight animals (**Fig. 13A,B**). Evidence of tumoricidal activity could be first detected on day+6 after ACT (**Fig. 13A**). Animals with complete tumor regression remained tumor free for only seven to 13 days (**Fig. 13A**). To assess viability and distribution of administrated FRβ CAR T cells, bioluminescence imaging *in vivo* was performed on day+2/9/18 after ACT. Results revealed that the CAR T cells homed specifically to the tumor site **(Fig. 13C**). Previous reports have suggested that CAR T responses in syngeneic mouse models are very short-lived, resulting in modest therapeutic responses. Indeed, our bioluminescence data (**Fig. 13C**) indicated a relatively transient accumulation of CAR T cells intratumorally. It has been suggested that the use of CAR T cells engineered to express T-cell-supportive factors, notably interleukin 12 (IL-12) or -15 (IL-15), may improve CAR T longevity and effector functions. To test this, we constructed FRβ CAR T cells constitutively expressing IL-15. Treatment of established B16 tumors in C57BL/6NRj mice by adoptive transfer of FRβ IL-15 CAR T cells resulted in complete tumor regression in two of eight animals, and three other animals showed anti-tumor responses (**Fig. 13A**). Hence, treatment with both FRβ IL-15 CAR T cells and FRβ CAR T cells resulted in delayed tumor progression compared to the control CAR T cell group, and it was demonstrated that CAR T cell therapy significantly improved survival ($p=0.0113$ for FRβ CAR T cell group and $p<0.001$ for FRβ IL-15 CAR T cell group; **Fig. 13B**).

A main challenge during this experiment was that a significant fraction of animals got very sick and lost over 20% of their initial weight within a very little time period (*data not shown*).

Of note, toxicity was observed in all irradiated mice receiving CAR T cells, including controls, suggesting that the toxic effect was not fully attributable to FRβ targeting *per se*. Previous experiments have shown that non-irradiated mice receiving FRβ CAR T cells, as well as non-tumor-bearing mice treated with irradiation and CAR T do not develop symptoms (*A. Tveita, unpublished results*). Hence, toxicity appears to be attributable to the combined presence of irradiation, CAR T transfer and a significant tumor burden. Although the underlying mechanisms have not been determined, the effect of fluid resuscitation in the initial phase following therapy suggests that symptoms might relate to CRS-like pathology.

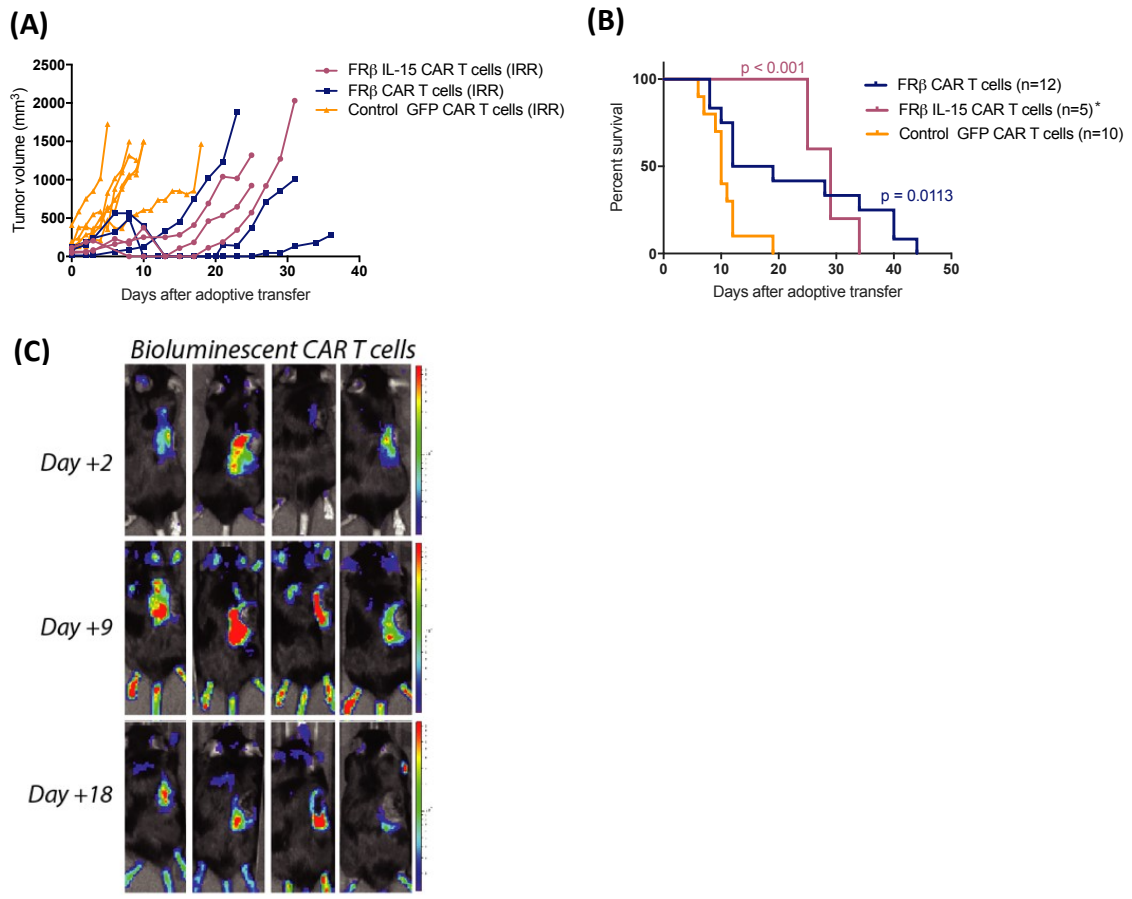

Figure 13. Adoptive transfer of FRβ-specific CAR T cells into mice with established murine melanoma *in vivo*. C57BL/6NRj mice were challenged with murine melanoma cells and adoptively transferred with *in vitro* generated CAR T cells when tumors reached a diameter of five mm. One day prior to adoptive T cell therapy (ACT), animals received 4Gy of whole-body irradiation. n=8 per treatment group. **(A)** Tumor volume measurements. Data for only a few representative animals of each group is shown. **(B)** Survival curves of tumor bearing mice at indicated number of days following ACT. * 3 animals were excluded due to death not related to CAR T cell therapy. **(C)** Bioluminescence images**.** Representative bioluminescence imaging data showing distribution of FRβ CAR cells at indicated days after ACT. Graphs show average dorsal luminescence signal.

3.4.2. Adoptive transfer of FRβ-specific CAR T cells show anti-tumor effect against established murine colon carcinoma (CT26) *in vivo*

Treatment of established CT26 tumors in Balb/c mice by adoptive transfer of FRβ IL-15 CAR T cells resulted in complete tumor regression in four of 12 animals, and partly tumor regression in four other animals (**Fig. 14A**). First evidence of anti-tumor activity was detected on day+14 post ACT (**Fig. 14A**). One of these animals remained tumor free even after four months (**Fig 14B**), whereas in two animals the tumor started to grow back after two weeks, and one animal had to be euthanized due to illness shortly after tumor regression (*data not shown*). No anti-tumor effects were observed in non-irradiated/irradiated animals without transferred CAR T cells and in the group adoptively transferred with control CAR T cells (**Fig. 14A**). Moreover, animals treated with FRβ IL-15 CAR T cells showed a slowed tumor growth compared to animals not receiving T cells and animals receiving control CAR T cells (**Fig. 14A**). While FRβ CAR T cells without the IL-15 gene did not show improved survival, a significant difference in survival was observed in the group treated with FRβ IL-15 CAR T cells ($p<0.01$) (**Fig. 14B**). The state of health was assessed by general behavior, fur structure and weight loss. Notably, animals treated with FRβ IL-15 CAR T cells got sick a few days following ACT (**Fig. 14C**). Subcutaneous administration of Ringer's acetate solution in appropriate time intervals resulted in complete recovery rates.

In summary, FRβ IL-15 CAR T cell therapy has demonstrated response rates of over 60% in Balb/c mice bearing CT26 tumors.

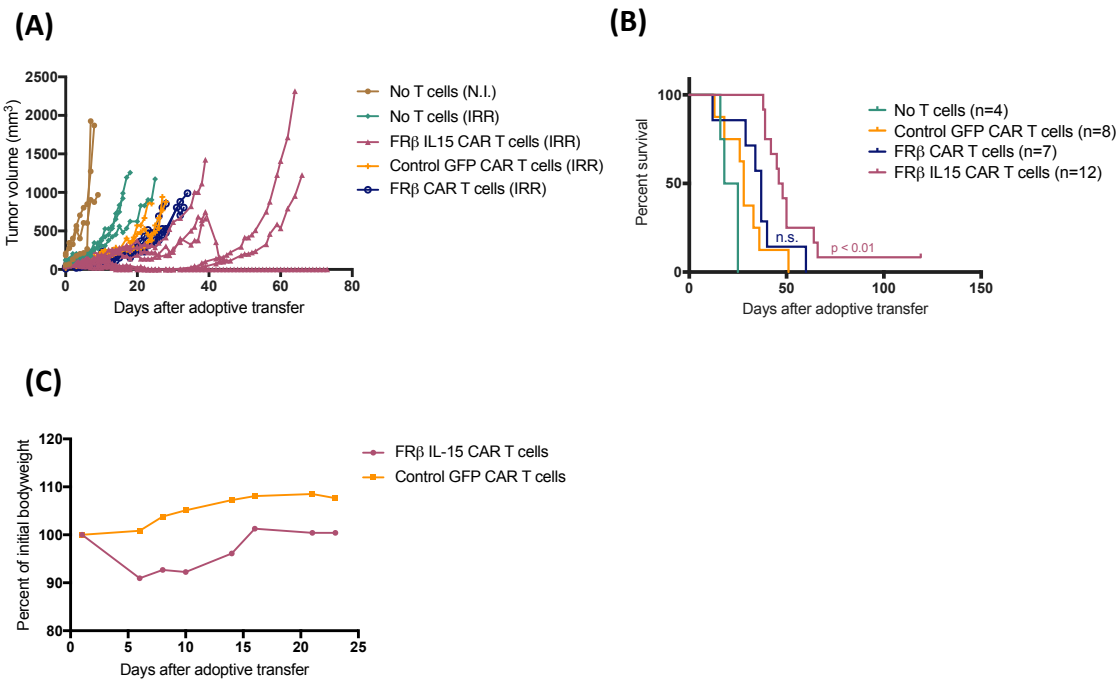

Figure 14. **Adoptive transfer of FRβ-specific CAR T cells show anti-tumor effect against established murine colon carcinoma *in vivo*.** Balb/c mice were challenged with murine colon carcinoma cells (CT26) (s.c.) and adoptively transferred with *in vitro* generated CAR T cells when tumors reached a diameter of five mm. One day prior to adoptive T cell therapy (ACT), animals received 4Gy of whole-body irradiation. n=4-12 per treatment group. **(A)** Tumor volume measurements. Data for only a few representative animals of each group is shown. **(B)** Survival curves of tumor-bearing mice at indicated number of days after ACT. **(C)** Body weight measurements at indicated number of days after ACT. Data for only one representative animal of each group is shown. n.s., not significant.

3.4.3 CAR T cell therapy in immunodeficient (Balb/c Rag1$^{-/-}$) mice

Although CAR T cells may mediate antitumor effects in their own right, several reports have indicated that the presences of highly activated T cells within the tumor site might elicit indirect immunostimulatory effects, e.g. via release of IFN-y [187]. Given our findings that FRβ CAR T cells induce pro-inflammatory cytokine release by TAMs (section 3.2, **Fig. 11**), our therapeutic approach might conceivably induce indirect activation of both innate and non-transgenic host adaptive immune cells. To assess whether anti-tumor effects of FRβ-specific CAR T cells are dependent on the presence of host adaptive immune cells, we performed tumor challenge experiments in Balb/c Rag1$^{-/-}$ mice. Balb/c Rag1$^{-/-}$ mice lack functional B and T lymphocytes because they are deficient in the recombination-activation gene (RAG). Mice were challenged with murine colon carcinoma cells (CT26). Treatment with FRβ IL-15 CAR T cells resulted in complete tumor regression in three of seven animals (**Fig. 15A**). Tumor regression was detectable two weeks after ACT (**Fig. 15A**). One of these three animals remained tumor free animal even after three months, while in the second animal the tumor

grew back after one month, and the third animal had to be euthanized because it was sick (*data not shown*). Treatment with FRβ CAR T cells without the IL-15 gene resulted in complete tumor regression in two of seven animals (**Fig. 15A**), but tumor started to grow back only a few days later, indicating that these CAR T cells had a less pronounced effect compared to FRβ IL-15 CAR T cells. No tumor regression, with one exception, was observed in the control group (**Fig.15A**). Even though FRβ IL-15 CAR T cells and FRβ CAR T cells showed anti-tumor effects, no significant difference in survival was observed compared to the control group (**Fig. 15B**). However, in animals treated with FRβ IL-15 CAR T cells, the therapy resulted in CRS-like responses in the animals, assessed by general behavior, fur structure and weight loss (**Fig. 15C**). Subcutaneous injection of Ringer's acetate solution showed high recovery rates.

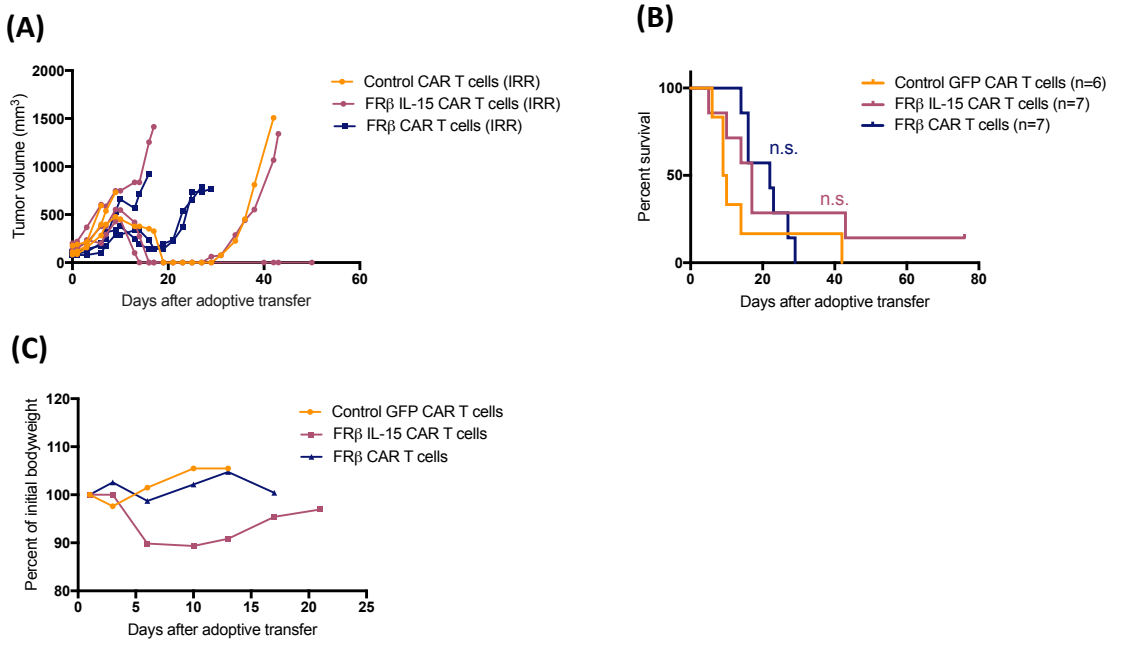

Figure 15. **Adoptive transfer of FRβ-specific CAR T cells show anti-tumor effect against established murine colon carcinoma in immunodeficient Balb/c Rag1$^{-/-}$ mice.** Balb/c Rag1$^{-/-}$ mice were challenged with murine colon carcinoma cells (CT26) and adoptively transferred with in vitro generated CAR T cells when tumors reached a diameter of five mm. One day prior to adoptive T cell therapy (ACT), animals received 4Gy of whole-body irradiation. n=8 per treatment group. **(A)** Tumor volume measurements. Data for only a few representative animals of each group is shown. **(B)** Survival curves of tumor-bearing mice at indicated number of days following ACT. **(C)** Body weight measurements at indicated number of days after ACT. Data for only one representative animal of each group is shown. n.s., not significant.

3.4.4 Adoptive transfer of FRβ-specific CAR T cells show anti-tumor effect against established Lewis lung (LL) carcinoma *in vivo*

Treatment of established LL tumors in C57BL/6NRj mice by adoptive transfer of FRβ IL-15 CAR T cells resulted in tumor regression in five of eight animals (**Fig. 16A**). Anti-tumor effects were detected on day+19 after ACT. Three of these animals with tumor regression were euthanized on day+36 since skin at the tumor site did not regenerate, and in the two other animals the tumor grew back after a few days. Treatment with FRβ-specific CAR T cells without the IL-15 gene resulted in tumor regression in only one of eight animals (**Fig. 16A**), and this animal had to be euthanized due to lack of skin regeneration at the tumor site. Since the majority of animals in this group did not show anti-tumor effects, these results may indicate that FRβ CAR T cells without the IL-15 gene had a less pronounced effect compared to FRβ IL-15 CAR T cells. In the control group, incomplete anti-tumor regression was noted in two of eight animals (**Fig. 16A**), possibly related to non-specific immunostimulatory effects of the ACT treatment regimen. Even though FRβ CAR T cells (with and without the IL-15 gene) resulted in delayed tumor progression, and FRβ IL-15 CAR T cells yielding response rates of over 60%, no significant difference in survival was observed compared to the control group (**Fig. 16B**). As seen in other mouse tumor models, FRβ-specific CAR T cell treatment resulted in impairment of the normal health state of the animals (Fig. **16C**). Subcutaneous administration of Ringer's acetate solution in appropriate time intervals showed high recovery rates

In summary, these results demonstrate that FRβ IL-15 CAR T showed anti-tumor effects, but no significant difference in survival compared to control group, with spontaneous regression of tumors in two of the control-treated mice. Lack of skin regeneration at the tumor site following treatment also presented a major challenge in the use of this mouse tumor model.

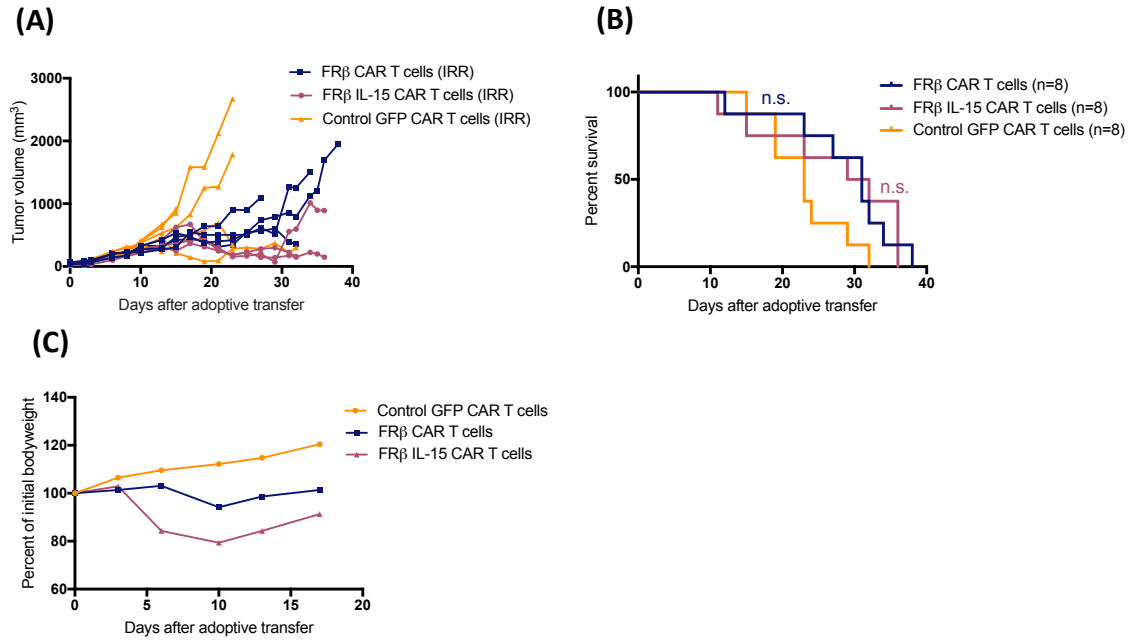

Figure 16. **Adoptive transfer of FRβ-specific CAR T cells show anti-tumor effect against established Lewis lung carcinoma *in vivo*.** C57BL/6NRj mice were challenged with Lewis lung carcinoma cells (LL) and adoptively transferred with *in vitro* generated CAR T cells when tumors reached a diameter of five mm. One day prior to adoptive T cell therapy (ACT), animals received 4Gy of whole-body irradiation. n=8 per treatment group. **(A)** Tumor volume measurements. Data for only a few representative animals of each group is shown. **(B)** Survival curves of tumor-bearing mice at indicated number of days following ACT. **(C)** Body weight measurements at indicated number of days after ACT. Data for only one representative animal of each group is shown. n.s., not significant.

3.4.5 Adoptive transfer of FRβ-specific CAR T show anti-tumor effects against established murine myeloma (MOPC315.4) *in vivo*

Treatment of established murine myeloma cells (MOPC315.4) bearing Balb/c mice with FRβ IL-15 CAR T cells resulted in complete tumor regression in one of eight animals, and this animal remained relapse free for one month (**Fig. 17A,B**). In the group treated with FRβ specific CAR T cells without the IL-15 gene, also one of eight animals showed complete tumor regression, and was tumor free even after three months (**Fig. 17A,B**). First evidence of tumor regression was possible to detect on d+20 and d+12 after ACT in the group with and without the IL-15 gene, respectively (**Fig. 17A**). No signs of tumor regression were seen in the control group (**Fig. 17A**). Moreover, the immunotherapy with both variants of FRβ CAR T cells have shown evidence of slowing tumor growth in the majority of treated animals compared to animals receiving control CAR T cells, as well as a significant difference in survival was observed ($p<0.001$ for FRβ CAR T cell group and $p<0.01$ for FRβ IL-15 CAR T cell group; **Fig. 17B**).

On the other hand, statistically difference in tumor growth rate and survival between the two FRβ CAR T cell groups were not detected (**Fig. 17A,B**). Consistent with observations from other

in vivo experiments, the therapy, and here especially FRβ IL-15 CAR T cell therapy, resulted in CRS-like responses in the animals, assessed by general behavior, fur structure and weight loss (**Fig. 17C**). Subcutaneous injection of Ringer's acetate solution resulted in complete recovery rates.

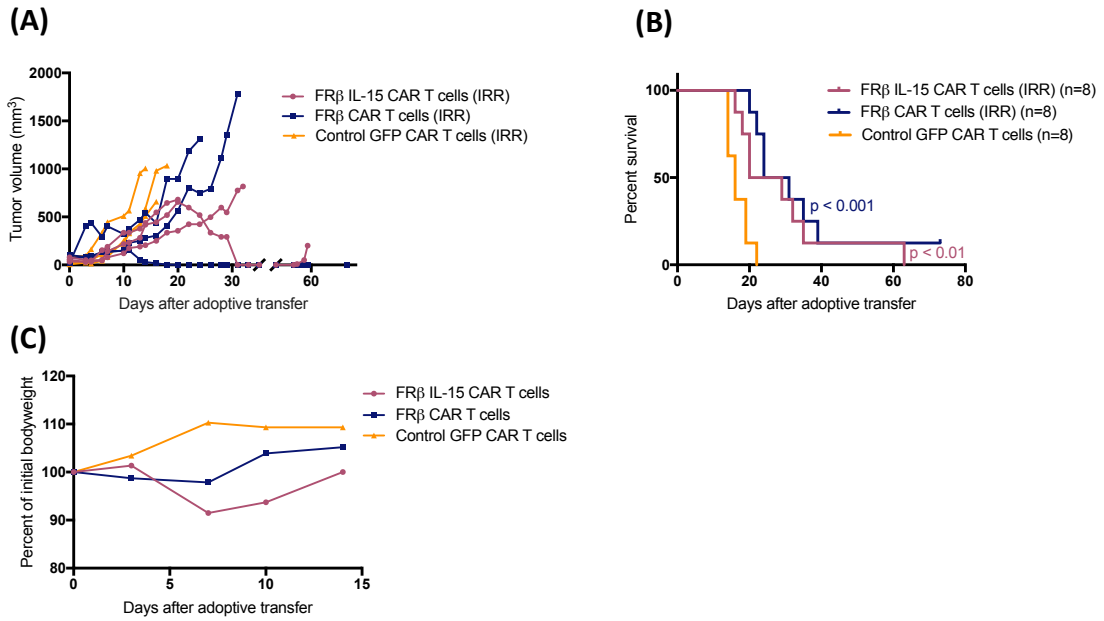

Figure 17. **Adoptive transfer of FRβ-specific CAR T cells into mice with established murine myeloma *in vivo*.** Balb/c mice were challenged with murine myeloma cells and adoptively transferred with *in vitro* generated CAR T cells when tumors reached a diameter of five mm. One day prior to adoptive T cell therapy (ACT), animals received 4Gy of whole-body irradiation. n=8 per treatment group. **(A)** Tumor volume measurements. Data for only a few representative animals of each group is shown. **(B)** Survival curves of tumor bearing mice at indicated number of days following ACT. **(C)** Body weight measurements at indicated number of days after ACT. Data for only one representative animal of each group is shown.

3.5 Generation of B16 FRβ knockout cell line by CRISPR/Cas9

Existing literature and knowledge about FRβ expression in healthy and malignant tissue strongly suggests a restricted expression confined to TAMs. This includes previous findings of Puig-Kröger et al. showing expression of FRβ on TAMs in melanoma and breast adenocarcinoma [154]. No FRβ expression was observed on *in vitro*-cultured tumor cells, and in functional *in vitro* assays, as well as no direct killing of tumor cells by FRβ-specific CAR T cells was noted (*A. Tveita, unpublished observations*). Nonetheless, there is a theoretical possibility that ectopic FRβ expression could occur during *in vivo* tumor growth. To exclude the possibility of a direct effect of our CAR T cells against tumor cells, we generated a variant of B16 cells deficient in the encoding gene using CRISPR/Cas9 technology. After transfection of B16 cells with PX458 expression vector containing the annealed sgRNA oligos, successfully transfected cells were sorted and cultured. Given the lack of detectable constitutive expression of the gene, screening of clones required gene sequencing to identify biallelic missense/nonsense mutants. DNA was extracted from the cells, and the DNA fragment spanning the intended sgRNA target sites amplified by polymerase chain reaction (PCR). Length of PCR products was checked on a 2.5% agarose gel (**Fig. 18**). Only DNA fragments with a similar length as the wild type DNA fragment (260 base pairs) were used for cloning into the plasmid vector pCR™-Blunt II-TOPO® followed by Sanger sequencing. Sequencing of multiple copies of each gene fragment per clone revealed that clone 3 (B16c3) harbored deletions resulting in a premature stop codon (**Fig. 19A-C**), and that clone 7 (B16c7) had a large deletion after the initiation site in one allele, and a one base pair-insertion on the other, both resulting in a non-functional gene (**Fig. 19D-F**). B16c3 and B16c7 were thus selected for further culturing.

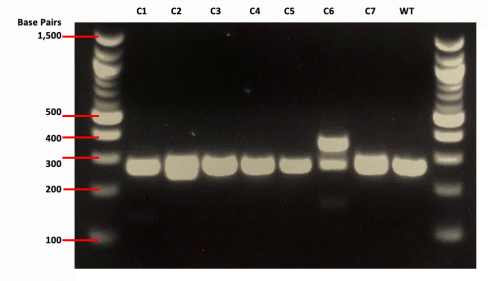

Figure 18. **Agarose gel electrophoresis (2.5% agarose) of PCR amplified products using primers specific for the DNA fragment which Cas9 is binding to.** B16 cells were transfected with PX459 expression vector containing the annealed sgRNA oligos. Successfully transfected cells were sorted and cultured. DNA was extracted from the cells and DNA fragment of interest amplified by PCR using 5'GGTCTCCCTAACTTTAGCTCCC3' and 5'GTGTGTCTGGCTGCTCACTAAC3' as forward and reverse primer, respectively. Length of PCR products was determined on a 2.5% agarose gel. The length of the wild type (wt) fragment using these primers is 260 base pairs. With the exception of c2 and c6, all other clones showed a similar fragment as the wt.

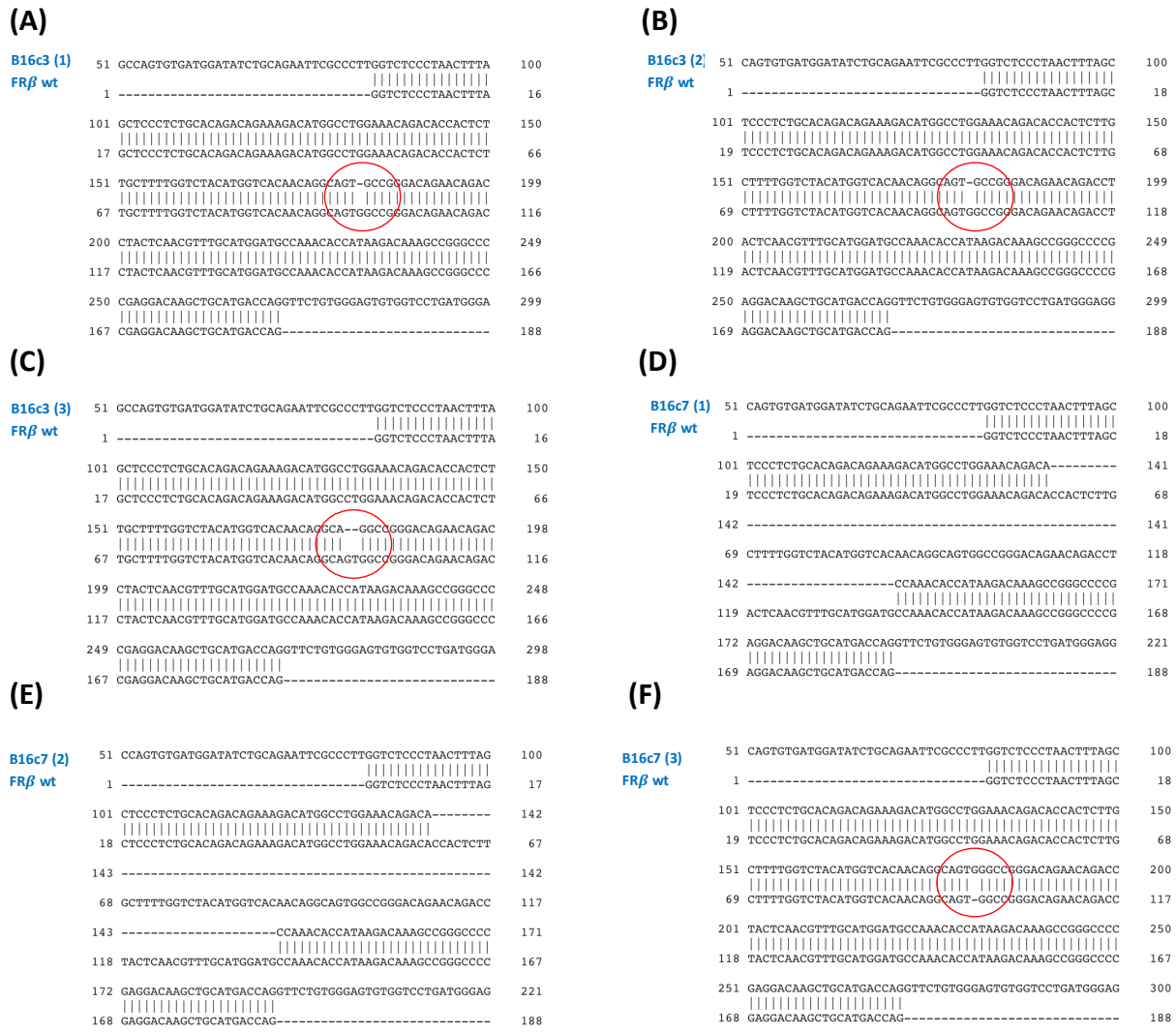

Figure 19. **Sequence alignment of wild type FRβ sequence with sequences of clone 3 and 7.** PCR products showing a similar length as the wild type DNA fragment on the 2.5% agarose gel were cloned into the plasmid vector pCR™-Blunt II-TOPO® followed by chemical transformation into chemically competent TOP10 *E. coli*. For each PCR product, three TOP10 transformants were expanded, plasmid DNA isolated by miniprep purification, and submitted for Sanger sequencing. **(A-C)** Transformants of the B16c3 cell line show deletions (encircled in red) that lead to a premature stop codon resulting in a non-functional FRβ protein. **(D-E)** Transformants one and two of the B16c7 cell line have a large deletion after the initiation site resulting in a non-functional FRβ protein. **(F)** Clone 3 of the B16c7 cell line had an insertion (encircled in red) leading to a premature stop codon.

4 Discussion

4.1. FRβ-specific CAR T cell s mediate indirect killing by re-polarizing TAMs *in vitro*

Macrophages are a highly plastic cell type that serve as effectors of both innate and adaptive immune responses. They further have been shown to influence tumor growth and progression depending on their polarization- and activation state dictated by the microenvironment [1, 59, 60, 67]. Recent studies showing that the phenotype of intratumoral macrophages is correlated with survival rate and response to therapy [64-66, 145], have sparked an increased interest in macrophages as potential therapeutic targets. Over the past few years there has also been a growing appreciation of the role of $CD4^+$ T cells in anti-tumor responses [72, 134-138, 150]. Previous work from our group has shown that indirect immune responses triggered by T cell-mediated re-polarization of TAMs are efficient in the elimination of cancer [138] Based on this, we hypothesized that CAR T cell targeting of antigens specifically expressed by TAMs would lead to TAM re-polarization with subsequent cancer elimination.

Historically, $CD8^+$ cytotoxic T lymphocytes (CTLs), due to their ability to initiate strong, cell-contact-dependent killing of target tumor cells, have been the main focus of CAR T cell therapy [188]. Nonetheless, there is a growing appreciation that $CD4^+$ T cells harbor effector functions that may also be of benefit in therapeutic immune responses against cancer. This work highlights a novel mechanism by which the helper function of $CD4^+$ T cells can be harnessed to elicit mechanistically distinct effects of potential therapeutic relevance.

In 2017, the FDA in the U.S. approved ACT using CD19-specific CAR T cells for the treatment of B-lymphoid malignancies [116-120]. CAR T cell therapy has shown extraordinary results in the management of relapsed/treatment-refractory B-cell malignancies, giving hope to a group of patients that previously had exhausted their treatment options [189]. However, application of CAR T cell in the setting of solid tumors has proven more challenging. Main obstacles include a paucity of appropriate tumor-specific antigens, and difficulties in facilitating CAR T cell migration to the tumor site and survival in the hostile microenvironment of the tumor. Hence, there is a large and currently unmet need for refinement of CAR T treatment for this form of therapy to be effective in most types of cancer.

As an alternate treatment strategy, we are exploring the potential of targeting immune cells within the tumor rather than the tumor cells themselves. Conceivably, this might circumvent the problems with conventional CAR T cell therapy relating to tumor-specific antigens in solid

tumors, and, to a greater extent harness the immunomodulatory effects of CAR T cells rather than their direct cytotoxic effector functions. Furthermore, by re-polarizing the TAMs we also hope to shift the microenvironment from a suppressive to a more pro-inflammatory state, thus actively counter-acting intratumoral immunosuppression.

In this thesis, seeking proof-of-concept for this strategy, FRβ was chosen as target because it has been reported to be marker for the anti-inflammatory M2-like TAMs both in various mouse tumor models such as ovarian carcinoma and C6 glioma [178, 190, 191], as well as in human solid tumors, including breast adenocarcinoma, melanoma, pancreatic cancer, head and neck squamous cell carcinoma, colon adenocarcinoma and prostate adenocarcinoma [154, 155, 177, 192, 193]. The restricted expression in mouse macrophages makes this an attractive model antigen, although a possible future utilization in humans would require an extensive characterization of off-site expression in healthy tissues and circulating monocyte subsets. With increasing knowledge about the molecular characteristics of TAMs, it is likely that new target structures with favorable expression patterns will emerge. As a negative control differing only in the nature of the targeting unit, CAR T cells containing the scFv fragment of the antibody M315 were engineered. M315 is a monoclonal IgA myeloma protein secreted by the murine MOPC315 myeloma cells, and not thought to be expressed on nor secreted by any other cell type than MOPC315 myeloma cells. This antibody was chosen based on its availability in the lab, and the fact that it has undergone extensive characterization and shows a well-defined binding specificity. Previous reports differ in their choice of negative controls, with some studies utilizing only mock-transduced T cells containing no CAR element [182-184, 186].

Our results showed that co-incubation of $CD8^+$ FRβ CAR T cells with M2-like TAMs *in vitro* resulted in a significant decrease in macrophage numbers rather than functional re-polarization (**Fig. 9**), results which are in agreement with conventional thinking about the mechanisms of action of CAR T cells. While depletion of TAMs by this means might counteract intratumoral immunosuppression, and conceivably might induce therapeutically relevant anti-tumor immune responses, we opted to focus this work on $CD4^+$ FRβ CAR T cells, which display a different functional profile, and which have been less extensively studies. Of note, other members of our group have performed side-by-side comparison of ACT using pure $CD4^+$

vs mixed $CD4^+/CD8^+$ FRβ CAR T cell preparations, and found the former to be significantly more effective (*A. Tveita, unpublished results*).

In accordance with previous findings of our group, demonstrating that Th1 CD4+ T cell could re-polarize TAMs towards the M1-like state [138, 164], our present results confirmed that FRβ-specific $CD4^+$ CAR T cells but not control M315 CAR T cells had the ability to re-educate TAMs and thus mediate tumor cytotoxicity by re-polarized TAMs *in vitro* (**Fig. 12A**). These results further confirmed that cognate T cell-macrophage interaction is crucial for the re-polarization leading to macrophage-mediated tumor killing [150]. Re-polarized macrophages secreted significantly higher amounts of pro-inflammatory cytokines (**Fig. 11**) and NO (**Fig. 12C**) compared to unstimulated macrophages and macrophages co-cultured with control CAR T cells. Again, these findings are consistent with previous results from our group [164]. NO secreted by activated macrophages diffuses into surrounding tumor cells where it can react with superoxide to generate the potent and toxic secondary metabolite peroxynitrite, resulting in the induction of tumor cell apoptosis. By inhibition of iNOS activity with 1400W, macrophage-mediated killing was completely abrogated (**Fig. 12B**), indicating that the anti-tumor effect triggered by FRβ-specific CAR T cells *in vitro* is dependent on iNOS activity.

The efficiency of tumor killing was dependent on the retroviral gene transfer of the CAR construct into T cells and on the activation state of the macrophages. Many steps in the protocol including the production of virus, the purification and activation of T cells from spleen, the purification and differentiation of macrophages from bone marrow and the transduction of T cells had to be extensively optimized. Low transduction efficiency of T cells resulted in low killing rates of cancer cells, indicating an incomplete activation- and re-polarization of TAMs. These results are consistent with findings from other groups that CAR T cell anti-tumor effector functions are regulated by CAR T cell density and the expression level of target antigen [194, 195].

In all *in vitro* assays, classically activated (M1) macrophages were used as positive controls. While this is not representative of the real-world situation in most tumors, it provides a benchmark for the types of tumoricidal responses that can be elicited through effective re-polarization of TAMs. Our *in vitro* results show that CAR T cell re-polarized TAMs killed MOPC315.4 myeloma cells with comparable, or in some cases, even better efficiency than classically IFN-γ/LPS activated M1 macrophages (**Fig. 12A**). Given that this form of macrophage-mediated killing is non-discriminatory, M1-like macrophages are capable of

eliminating neighboring cells irrespective to their antigen expression; a phenomenon referred to as bystander killing. In a clinical setting, this feature could be relevant for solid tumors that are comprised of highly heterogenous cell populations in a complex and dynamic microenvironment.

In summary, the main objective was to verify the ability of FRβ-specific CAR T cells to promote elimination of cancer cells by re-educating TAMs towards the anti-tumor phenotype. Our findings demonstrate that FRβ CAR T cells mediate efficient and specific targeting of TAMs in *in vitro* co-cultures and cytotoxic assays. Up-regulation of activation markers, an increased production of pro-inflammatory cytokines, and efficient target cell lysis were observed. *In vitro* re-polarized macrophages show strong anti-tumor effects independent of presentation and/or secretion of a tumor-specific antigen. Mechanistically, these effects can be explained by induction of iNOS expression and activity, with release of NO, in turn triggering apoptosis in neighboring tumor cells. Future refinement, e.g. studies utilizing directly *ex vivo*-isolated TAMs or crude single-cell tumor suspensions is likely to provide further insight into the efficacy of these processes in a more physiological system.

4.2 Indirect tumor elimination mediated by FRβ-specific CAR T cells *in vivo*

Patients that receive ACT are lymphodepleted with chemotherapy alone, or in combination with whole body irradiation prior to T cell transfer. This preparative treatment leads to a transient decrease in lymphocytes and other immune cells, thus creating homeostatic space for the CAR T cells which facilitates their survival and functionality in the host [115]. In addition, these treatments can cause immunogenic cell death of tumor cells leading to an induction of anti-tumor immune responses.

In contrast to naïve T cells, *in vitro* generated CAR T cells are already activated and show an up-regulated expression of surface markers for migration when they are transferred into the patient, thus bypassing the need for re-activation in the lymph node. Nonetheless, previous studies from our group with adoptive transfer of bioluminescently labeled T cells has confirmed that recipient mouse preconditioning, most efficiently mediated by sublethal irradiation, was required for successful engraftment of transferred T cells. To fully assess the therapeutic potential of FRβ-specific CAR T cells in established solid tumors, adoptive transfer experiments were performed in immunocompetent Balb/c and C57BL/6NRj mice and

immunodeficient Balb/c Rag1$^{-/-}$ mice when the tumor reached a diameter of five mm. This provides a challenging starting point for therapeutic interventions, but provides a model of advanced stage, established cancer, which constitutes the clinical scenario in which CAR T therapy might be considered. Secondly, a certain tumor size was necessary to ensure a sufficient infiltration of macrophages into the tumor. On the other hand, tumor burden should still be small (∼five mm) so that T cell-mediated effects can be observed within the very narrow timeframe before tumor growth to excessive size. The model system dictates that responses must occur before the tumor size reaches ≥20 mm, which was established as the humane end point. This is especially important for rapidly growing cancers (e.g. B16 melanoma).

Adding to the challenges of our *in vivo* model, tumor growth is likely to occur largely unperturbed for some days after ACT, since transferred T cell need some time to home to the tumor site and once there to expand and exert their effector functions (*data not shown*). Strikingly, in our experiments anti-tumor effects were consistently observed only after one to two weeks post ACT. In contrast, our *in vitro* data shows an efficient re-polarization of TAMs by T cells within 24 hours (**Fig. 10F-K, 11, 12A**). These observations suggest that the net results of cellular interactions within the tumor are more complicated than in our *in vitro* setup, and likely involves numerous cell types. Key questions for further follow-up experiments include the kinetics and extent of CAR T migration throughout the tumor, the time needed for to reach and re-polarize macrophages, and the extent and dynamics of expression of the target structure on TAMs in the course of the experiment. Moreover, these findings might indicate that *in vivo* T cell mediated tumor killing is not only achieved by direct killing of tumor cells by re-educated TAMs but possibly also by the endogenous immune system which can be activated by cytokines, chemokines and other molecules released from (activated) immune cells in the tumor. Since re-development of the endogen immune system needs some time after lymphodepletion, this could be a possible explanation for delayed observations of anti-tumor effects. If this is the case, this would suggest that the use on non-lymphoablative preconditioning or preconditioning-free adoptive transfer approaches might potentiate and speed up anti-tumor immune responses. On the other hand, it is possible that effects of irradiation on the tumor microenvironment could be important to the ensuing immune response. Longitudinal flow cytometry, immunohistochemistry and cytokine profiling studies

of the tumor microenvironment are likely to cast further light on these issues, as is experiments performed using immunodeficient hosts.

By using syngeneic mouse tumor models (Balb/c and C57BL/6NRj mice) in our adoptive transfer experiments, the real-world situation in humans were reflected to a certain extent. However, there are clearly differences between mouse and human immunology [196], and in addition, the homogeneity of inbred mouse strains differs greatly from the heterogeneous human population. Therefore, extrapolation of findings from mouse models to humans harbors potential limitations and must be done with great caution.

One day prior to ACT, the animals received whole-body irradiation (4 Gy) to ensure a better survival and functionality of transferred CAR T cells, however, considering the hypothesized involvement of the endogenous immune system we also tried ACT without any pre-treatment (*data not shown*). In agreement with previous experience in the lab and previous reports [197, 198], no beneficial effect was seen as the tumors grew faster with no pretreatment, and the injected T cells showed a considerable delay in proliferation (*data not shown*). *In vivo* results demonstrated that adoptively transferred FRβ-specific CAR T cells led to a significant delay in tumor outgrowth in all mouse tumor models tested (compared to groups with adoptively transferred control CAR T cells or only irradiated animals with no T cells transfer), and in some cases complete tumor regression was observed. While most of the tumors eventually relapsed, these results appear striking, given the limited number of pre-existing reports of successful application of CAR T therapy in immunocompetent syngeneic models, with most showing only marginal survival benefits [199, 200]. In addition, treatment was effective in multiple unrelated models, indicating that the underlying mechanism of action has potential relevance in multiple types of cancer.

Our results indicate that intravenous injection of FRβ-specific CAR T cells led to homing and extravasation of CAR T cells at the tumor site, and activation and re-education of TAMs within the tumor with resulting lysis of tumor cells at sites of established tumors. However, in over 90% of the animals that showed complete tumor remission, the animals got a relapse after a short time lag. In addition, a significant number of mice had to be euthanized during the observation period following therapy, due to either an impaired health state or the lack of skin regeneration at the tumor site, specifically seen in Lewis lung carcinoma. Since tumor relapse was commonly observed, most likely due to limited survival of the transferred CAR T cells, or loss of effector functions. Indeed, activation-induced cell death (AID) occurring after

T cell activation is a pronounced feature of murine T cells in particular, and is commonly cited as a major reason for the limited effects of CAR T cells in syngeneic mouse models in previous studies [199, 201].

Repeated injections of CAR T cells, or optimization of the CAR T to improve longevity could be considered as strategies to mitigate such obstacles in future experiments.

Other explanations for limited duration of regression include outgrowth of residual tumor cells (e.g. cancer stem cells (CSCs)) that are resistant or survive CAR T cell therapy.

Lack of anti-tumor effects of CAR T cells or only incomplete tumor regression, as seen in our experiments, could also result from incomplete infiltration of CAR T cells into the tumor site, or/and that tumor areas with low TAM density could not sufficiently be re-directed into a tumoricidal environment. It is important to note that macrophage-mediated tumor-lysis is spatially limited and depended on short-lived secreted factors [164].

One major barrier against CAR T cell infiltration into solid tumors is the cancer associated stroma, which overexpresses receptors and ligands that obstruct T cells from passing this barrier [202]. Caruana et al. (2015) demonstrated that *in vitro* manipulated T cells lost expression of heparanase (HPSE; an enzyme that degrades heparan sulfate molecules found in the extracellular matrix), compared to freshly isolated T cells, and that this lack of HPSE reduced the ability to penetrate the tumor endothelium [203]. By engineering CAR T cells expressing HPSE, higher infiltration rates were observed [203]. Furthermore, the unbalanced secretion of chemokines and cytokines from the tumor mass results in insufficient homing of T lymphocytes and other immune cells to the tumor site [202]. Other major barriers for efficient anti-tumor responses are tumor-intrinsic mechanisms and the inhibitory tumor microenvironment [202, 204]. A hypoxic microenvironment, the lack of efficient levels of nutrients and glucose, the limited availability of amino acids (AA), and release of prostaglandin E2 (PGE2) by tumor cells and macrophages are all factors that inhibit T cell proliferation and cytokine production, and thus an effective anti-tumor effect. Presence of T_{regs} that also infiltrate the tumor further suppress T cell responses by secretion of TGF-β [202, 204, 205]. Since it has been shown that the composition of the tumor microenvironment differs greatly between different cancer types, but also among patients/animals bearing the same class of malignancies [143, 144], this represents an explanation why anti-tumor response were not always seen in our experiments. Moreover, incomplete tumor regression and/or tumor relapse can also result from tumor cell heterogeneity. Due to genomic instability, new tumor

cell variants are constantly formed, which could lead to an increased secretion of immunosuppressive cytokines, chemokines and molecules counteracting the immunomodulatory effects of CAR T cells. To boost the potency of CAR T cells, the use of CAR T cell therapy in combination with immunomodulatory drugs such as check point inhibitors has proven to be a promising strategy. Check point inhibitors (e.g. anti-PD-1/PD-L1 and anti-CT2A) reverse the immune-checkpoint blockage that obstructs an active immune response within the tumor [206]. Therefore, it should be investigated whether the use of checkpoint inhibitors in combination with ACT of CAR T cells can result in better tumor regression rates and lower the relapse rate in our mouse models.

Given the observed one to two-week delay in effect of treatment we hypothesized an involvement of the endogenous immune system. To further elucidate the mechanism of FRβ-specific CAR T cells ACT experiments were performed in immunodeficient Balb/c Rag1$^{-/-}$, lacking mature and functional B and T lymphocytes. The experiments performed to date, and only in a relatively small number of mice, indicate that anti-tumor effects triggered by FRβ-specific CAR T cells were seen in the absence of a functional adaptive immune system in the host but did not result in significant better survival rates compared to the control group (**Fig.15 A,B**). In comparison to wild type Balb/c mice, the anti-tumor effects were less pronounced, possibly indicating a functional role for endogenous adaptive immune cells in this mechanism of tumor killing *in vivo*. No T and B lymphocytes can be recruited to the tumor site resulting in an altered secretion level of cytokines, chemokine- and immunoglobulins. However, the therapeutic effect was not completely abolished, as we do see mice with tumor regression in this mouse model as well. These results indicate that although host adaptive immune cells may act in synergy with TAM-directed CAR T cell therapy, they do not appear to be critically required. Additional experiments with larger cohorts of mice, and experiments involving reconstitution of recipients with non-transduced T cells are required to strengthen the conclusions of these studies. Although lacking B and T lymphocytes, Balb/c Rag1$^{-/-}$ mice do have functional natural killer (NK) cells, a cell type known to have important to have anti-tumor effects [207]. NK cells are known to be activated by IL-15 and could contribute to the anti-tumor effects. In order to evaluate the impact of NK cells in this mechanism of tumor killing, adoptive transfer experiments in Rag2$^{-/-}$ γc$^{-/-}$ mice; which are also devoid of NK and natural killer T (NKT) cells, should therefore be included in future studies. To investigate the

functional relevance of chemokines, adoptive transfer experiments might also be performed in knockout mouse models deficient in candidate chemokine receptors, or by the use of blocking/neutralizing mAbs.

With the therapeutic potential of CAR T cell therapy comes a risk of severe side effects including unforeseen cross-reactivity or off-site targeting of healthy tissue, the cytokine release syndrome (CRS) and tumor lysis syndrome [115, 121, 123-129]. CRS represents a major concern in current CD19 CAR T therapy, but its occurrence may at the same time serve an indication of successful target engagement, signifying that the treatment is working. Giavridis et al. (2018) [208] and Norelli et al. (2018) [209] found that monocytes and macrophages, and not CAR T cells, constitute the main source of IL-1 and IL-6, the cytokines most commonly implicated in the pathogenesis of CRS. Administration of an anti-IL-6 receptor antibody (tocilizumab), and, in preclinical studies, the use of CAR T cells that constitutively produce the IL-1 receptor antagonist (IL-1RA), have been shown to mitigate CRS toxicity quite efficiently [129, 208]. These findings notwithstanding, it is clear that our current understanding of the CRS phenomenon is limited, and it seems likely that such side effects can be further avoided with increasing insight into the pathogenesis of this syndrome, and with increasing sophistication of T cell engineering.

In mice, the state of health after the adoptively transfer of CAR T cells was assessed by general behavior (reduced activity), fur structure and weight loss. Only a handful of studies on the use of CAR T cell syngeneic mouse models have been published to date, and they do not include information about side effects or interventions performed to treat such occurrences. Hence, there was little pre-existing information to guide the symptomatic management of animals following CAR T therapy. Since the treatment with tocilizumab and IL-1RA showed good effects in humans, murine homologues of these agents were evaluated in our mouse models. Anti-IL-6 receptor antibody treatment did not show a positive effect on survival in our models and furthermore, was found to have a negative impact on FRβ-specific CAR T cells mediated tumor killing (*data not shown*). In clinical practice, Ringer's acetate is administrated to manage fluid losses and to prevent isotonic dehydration in patients with severe burn injuries and sepsis [210, 211]. With this knowledge, we wanted to investigate whether animals that develop CRS-like symptoms shortly after the administration of CAR T cells show a better survival when dehydration (due to illness) was counteracted with the administration of

Ringer's acetate solution. Our findings demonstrate that good monitoring of the animals and injection of Ringer's acetate solution at needed timepoints significantly improved the animals' condition and resulted in very high recovery rates. Subcutaneous administration allows for a controlled and slow absorption of the fluid from beneath the skin into the blood stream of the animals.

Many pre-clinical studies have been performed to evaluate the effect CAR T cell therapy against various solid tumors, although the vast majority have been performed using human xenograft tumors and human CAR T cells in severely immunocompromised mice. In such models, limited cross-species reactivity of cytokines and surface receptors results in limited interaction between CAR T cells and host-intrinsic cells. Also, the lack of a lymphoid compartment removes the need for host preconditioning. In summary, this conventional experimental approach fails to recapitulate the interactions that underlie the tripartite interaction between tumor cells, stroma and CAR T cells. Hence, key obstacles to implementation of CAR T therapy in solid tumors are not reflected by such models. In our studies we have utilized fully immunocompetent mice, and we would argue that this provides a more clinically relevant framework for evaluation of CAR T therapy.

Most studies performed so far have concentrated on engineering CAR T cells specific for tumor-restricted epitopes, including CEA CAR T cells/5E5 CAR T cells against pancreatic cancers, Claudin18.2-specific CAR T cells against gastric cancer, L1-CAM CAR T cells against ovarian cancer and EGFR CAR T cells against lung cancer [182-186]. Our *in vivo* results demonstrate that adoptively transferred FRβ-specific CAR T cells triggered anti-tumor effects by T-cell-mediated re-polarization of TAMs, indicated by slowed tumor growth and even complete tumor regression. Moreover, anti-tumor effects were not restricted to only one cancer type. Therefore, we here present a novel mechanism of tumor killing which is independent on the expression of tumor-restricted epitopes or secretion of tumor-specific antigens, but on the other hand is highly dependent on direct T cell-TAM interaction within the tumor, and thus requires macrophage infiltration within tumors. Side effects like CRS were treated with subcutaneous administration of Ringer's Acetate and resulted in very high recovery rates. Our findings provide initial proof-of-concept for a novel form of CAR T therapy with a unique mode of action, and provides a basis for further refinement of the treatment regimen. A major observation is the potential for eliciting active immunomodulation of the

tumor microenvironment by harnessing the unique effector properties of $CD4^+$ T cells. Additional mechanistic studies, and preclinical studies using human cells are needed to determine the clinical applicability of this form of therapy.

5 FUTURE PERPECTIVES

Direct/indirect tumor killing and the impact of macrophages

Although our results indicate that indirect tumor cell killing mediated by FRβ-specific CAR T cells was observed both *in vitro* and *in vivo*, we cannot completely exclude the ability of direct killing of tumor cells by the T cells. We have therefore generated a FRβ knockout variant of B16 using a CRISPR/Cas9 approach. This will enable us to evaluate whether indirect macrophage-mediated cytotoxic response elicited by T cells is the main contributor for sufficient anti-tumor response. Conversely, demonstration that experimental depletion of TAMs abrogates the effect of FRβ-specific CAR T cells would be useful in confirming the hypothesized mechanism-of-action.

Alternate targets

The choice of FRβ as the target antigen was made based on its reported overexpression in TAMs in various tumor models, as well as the existence of high-affinity scFv fragments. Other surface receptors have been reported to be selectively expressed by TAMs, and new potential targets are likely to emerge with advances within the field. The herein explored treatment approach should be applicable to such alternate targets, and might yield therapeutics with improved selectivity and efficacy. Studies are underway in our group to develop and test CAR T-based agents targeting such alternate TAM markers. Moreover, other tumor-infiltrating cell types could conceivably be targeted in a similar manner.

Memory potential of CAR T cells

To test whether mice that displayed complete tumor regression and showed a disease-free survival have developed a long-term memory, immune responses against tumor re-challenge in these animals could be of interest to investigate.

Incomplete tumor regression and relapse rate

Even though eradication of established tumors mediated by FRβ-specific CAR T cells was observed, the majority of the animals got a relapse after a short time lag. Therefore, it is of great interest to investigate whether another round of ACT will improve long-term outcome. Another point to think of could be the optimization of the CAR construct. By modifying the

hinge length and signal transduction domains, as well as by improving scFv affinity, CAR T cell functionality, persistence and target affinity could be greatly improved. However, a higher target affinity increases the risk of cytolysis of normal cells expressing FRβ in lower levels, and must therefore be taken into consideration. Our results suggests that an "armored CAR T" strategy utilizing IL-15 allows improved efficacy, at least in some models. Further studies are warranted to determine the mechanistic basis for the improved outcome with provision of IL-15. Additional cytokines, including IL-12 and IL-18 [107], have been suggested to similarly improve treatment efficacy in preclinical models. Exploration of different cytokines, and bioluminescence/flow cytometry studies to determine the longevity and growth kinetics of different armored CAR T preparations might assist in obtaining a treatment regimen with improved efficacy.

Impact of other immune cells

With regard to develop a novel immunotherapeutic treatment, it is important to verify how the tumor cells are getting eliminated. The impact of other immune cells must therefore be evaluated. To examine the distribution of various immune cells within the tumor, we further want to perform immunohistochemically analyses, with the aim to get a better understanding of why anti-tumor responses were incomplete or absent.

Our present results demonstrate that anti-tumor effects can be observed in immunodeficient Balb/c Rag1$^{-/-}$ mice, indicating that indirect killing mediated by of FRβ-specific CAR T cells is possible in the absence of mature CD8^{+} and other CD4^{+} T cells. However, other cells like NK cells, eosinophils or neutrophils may also play a more or less important role. To elucidate their roles in our treatment, we want to perform adoptive transfer experiments in other immunodeficient mouse models, such as Balb/c Rag2$^{-/-}$ γc$^{-/-}$ mice that are completely alymphoid.

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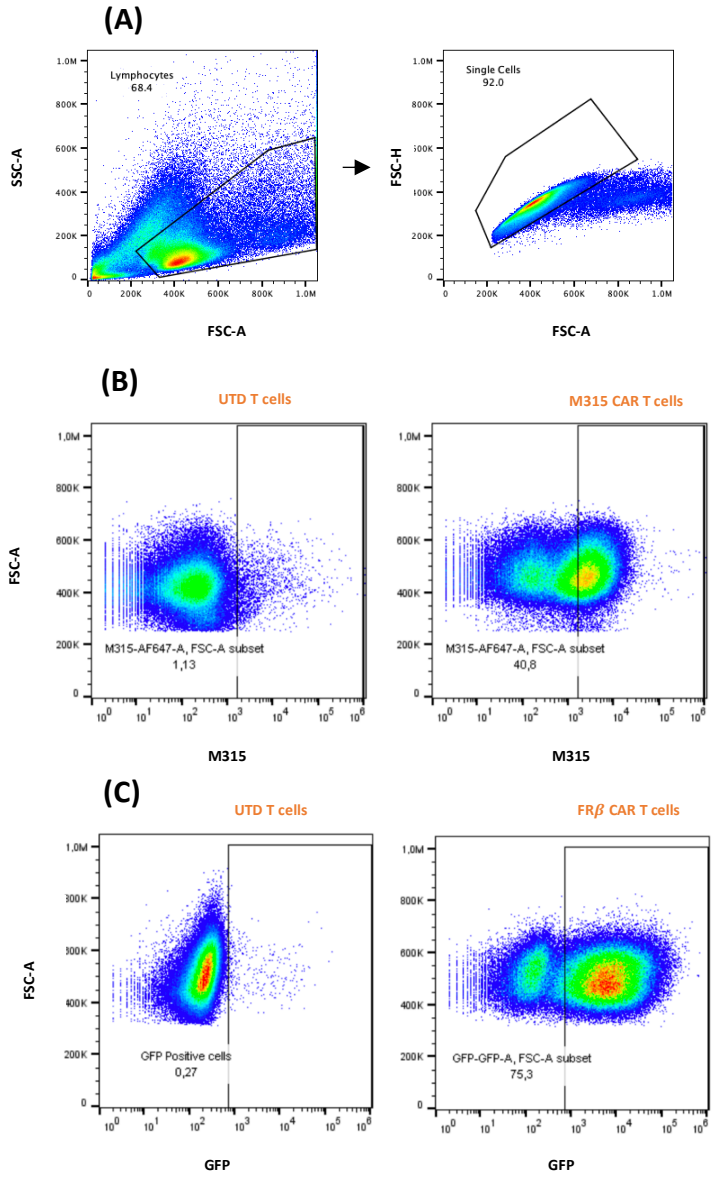
APPENDIX

Appendix 1



Appendix Figure A1. **Evaluation of retroviral transduction efficiency of M315 CAR T cells and FRβ CAR T cells by flow cytometry.** Two days prior to the transduction start, murine $CD4^+$ T cells were isolated from spleen. Naïve $CD4^+$ lymphocytes were isolated by positive selection with magnetically labeled microbeads (CD4 (L3T4). For activation, $CD4^+$ T cells were cultured on a plate pre-coated with anti CD3 and anti-CD28 in complete RPMI supplemented with 50 µM 2-Mercaptoethanol and 1% Insulin-Transferrin-Selenium. On the next day, murine IL-7 and IL-2 were added to enhance proliferation rate and cells were expanded for another 24 hours before they were used for retroviral transduction. Transduction efficiency was evaluated performing flow cytometry. **(A)** T cell population was defined on forward and side scatter. **(B,C)** Transduction efficiency was calculated based on T cells that were not transduced. For M315 CAR T cells the transduction efficiency was around 41% and for FRβ 75%. The figure shows data for only one representative experiment.

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