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Chimerické antigenní receptory a jejich využití pro léčbu hematologických malignit

Chimeric antigen receptors in the treatment of hematological malignancies

Diplomová práce

Vedoucí závěrečné práce: RNDr. Dominik Filipp, CSc.

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Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Abstract

Chimeric antigen receptors (CARs) are artificial molecules composed of an antibody derived antigen recognition domain which is fused with the signal transduction domain derived from the physiological TCR. CAR technology used to transduce patients T-cells and endow them with the specificity to a certain surface antigen, has been a major breakthrough in cancer immunotherapy in the last decade. This strategy has been most successful for treating hematologic malignancies. Various CAR approaches and applications are currently tested mainly in the United States where many clinical trials have been launched. In contrast, in the Czech Republic, there are only a few teams focused on this topic with no clinical trials going on.

During my work on this diploma thesis and in close collaboration with MUDr. Pavel Otáhal, PhD., who is working on implementation of CAR technology into the Czech clinics for the treatment of B-cell malignancies, individual functional CARs were prepared and tested. CAR expressing Jurkat T-cell lines were generated using a lentiviral vector transduction system. CAR functionality was determined by two different assays. We have shown that individual CARs are able to recognize the B-cell lineage specific antigens CD19 and CD20 and significantly up-regulate the activation molecule CD69 upon T-cell activation by co-cultivation with RAJI B cell used as a target. Individual CAR constructs also showed to be functional in the mouse thymoma cell line with NFAT-GFP reporter. Our ultimate goal, the preparation of a superCAR construct that would endow T-cell with dual specificity against both CD19 and CD20 antigens has not been accomplished yet and the work on this construct is still in the process.

Within the work frame we have prepared suitable conditions for further experimental testing of CAR technology *in vitro*. Future perspective of this work relates to the completion of the superCAR construct. We hopeful that that newly designed dual specificity CAR construction would prove efficient in preventing malignant B-cells that have lost the expression of one of the B-cell lineage specific antigens, in order to escape their detection. The CAR technology for cancer immunotherapy is a perspective therapeutic strategy worth of research in the Czech.

Key words: treatment of leukemia, T cells, TCR, chimeric antigen receptor, B cells, CD19, CD20, co-stimulation, immunotherapy

Abstract (CZ)

Chimérické antigenní receptory (CAR) jsou syntetické molekuly kombinující antigenní specifitu monoklonální protilátky se signalizací běžného T-buněčného receptoru. Těmito receptory jsou geneticky modifikovány lidské T-lymfocyty a ty jsou tak vyzbrojeny konkrétní antigenní specifitou. Tato technologie je průlomovou v oboru nádorové imunoterapie. Adoptivní transfer geneticky upravených T-lymfocytů byl doposud nejúspěšnější formou léčby hematologických malignit. To z toho důvodu, že nádory krve mají difúzní charakter, na rozdíl od pevných nádorů, které mají charakteristické imunosupresivní mikroprostředí. CAR technologie je v současné době nejintenzivněji studována ve Spojených státech, kde probíhají desítky klinických studií. Přesto, že se tato terapeutická metoda jeví jako velice perspektivní, v České republice se jí věnuje velmi omezený počet lidí a probíhající klinické studie neexistují.

Ve spolupráci s MUDr. Pavlem Otáhalem, který se tímto tématem zabývá a má velký zájem dostat tuto terapeutickou metodu do české klinické praxe, jsme připravili několik receptorů, jejichž samostatnou funkčnost jsme ověřili dvěma nezávislými metodami. Stabilní CAR-exprimující T-buněčné linie rozpoznávající B-buněčné antigenům CD19 a CD20 byly připraveny za pomocí lentivirů. T-buněčné linie exprimující jednotlivé konstrukty dokázaly rozeznat B-buněčné antigeny a došlo k jejich aktivaci bezprostředně po ko-kultivaci s cílovými Raji buňkami. Aktivace se projevila významným zvýšením exprese aktivační molekuly CD69. Funkčnost konstruktů jsme potvrdili dalším alternativním přístupem, a to prostřednictvím jejich exprese v myší thymomové buněčné linii obsahující NFAT-GFP reportér. Příprava superCAR konstruktu, jehož využití by zajistilo simultánní expresi CD19 a CD20 specifických receptorů v T-lymfocytech je zatím ve fázi přípravy.

V rámci mé diplomové práce jsem si osvojena základní metodiku pro přípravu chimérických antigenních receptorů a byla připravena půda pro budoucí *in vitro* a *in vivo* experimenty. V budoucnu bychom rádi dokončili přípravu superCAR konstruktu a prokázali, že jeho využití znemožní únik nádorových klonů, u kterých došlo ke ztrátě jednoho z B-buněčných povrchových antigenů. CAR technologie je perspektivní nádorovou imunoterapeutickou metodou, které by měl být v České republice věnován větší prostor.

Klíčová slova: léčba leukemie, T lymfocyty, TCR, chimerický antigenní receptor, B-lymfocyty, CD19, CD20, kostimulace, imunoterapie

List of abbreviations

AICD	activation induced cell death
ALL	acute lymphoblastic leukemia
allo-HSCT	allogeneic hematopoietic stem cell transplantation
AMP	ampicillin
AP-1	activator-protein 1
APC	antigen presenting cell
BCR	B-cell receptor
CAIX	carbonic anhydrase IX
CAR	chimeric antigen receptor
CLL	chronic lymphocytic leukemia
CTL	cytotoxic T-lymphocytes
CTLA-4	cytotoxic T- lymphocyte antigen 4
DAG	diacylglycerol
DAMP	damage associated molecular pattern
ECL	enhanced chemiluminiscence substrate
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FDA	Food and Drug Administration
GD2	disialoganglioside
GEF	GTP exchange factors
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony stimulating factor
HLA	human leukocyte antigen
HEK	human embryonic kidney
HIV	human immunodeficiency virus
iCaps9	inducible caspase-9
ICOS	induced co-stimulator
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukine
IP_3	inositol triphosphate
ITAM	immunoreceptor tyrosine activation motif
LAT	linker for activation of T-cells
LB	Luria-Bertani
LCK	lymphocyte specific tyrosine kinase
LTR	long terminal repeats
MAP	mitogen-activated protein
MRD	minimal residual disease

mTEC	medullary thymic epithelial cell
NFAT	nuclear factor of activated T-cells
NK cells	natural killer cells
NFκB	nuclear factor kappa B
NKT cells	natural killer T-cells
mAb	monoclonal antibody
PCR	polymerase chain reaction
PD-1	programed cell death protein 1
PEI	polyethylenimin
PLC-γ	phospholipase C-γ
PM	plasma membrane
pMHC	peptide-MHC
PTK	protein tyrosine kinase
PVDF	polyvinylidene fluoride
RAG	recombination activation enzyme
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	Src homolog 2
SIN	self-inactivating
SLP76	SH2-domain-containing lymphocyte protein of 76kDa
scFv	single chain fragment variable
TAA	tumor associated antigen
TCR	T-cell receptor
tEGFR	truncated epidermal growth factor receptor
TGF-β	transforming growth factor β
TIL	tumor infiltrating lymphocyte
TNF	tumor necrosis factor
TNRF	tumor necrosis receptor family
TRAIL	tumor necrosis factor-related apoptosis inducing ligand
Treg	regulatory T-cells
TSA	tumor specific antigens
VH	variable heavy
VL	variable light
VEGF	vascular endothelial growth factor
VSV-G	vesicular stomatitis virus glycoprotein
ZAP70	ζ-chain associated protein kinase of 70kDa

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1. Introduction

Cancer is one of the leading causes of death in the 21st century[1]. To a certain extent, this is due to fact that more than 100 different types of malignancies[2] are treated by largely non-specific treatment such as surgical removal, chemotherapy, radiotherapy or their combination. While these are inevitable for the elimination of the cancerous tissue, these approaches are associated with many negative side effects and are often insufficient (Hinrichs and Restifo, 2013). Another and attractive option for the treatment of these malignancies is to use the tremendous capacity of our immune system to recognize and remove the tumor. Tumor cells accumulate mutations and express tumor specific antigens (TSA) and tumor associated antigens (TAA) that should ensure their detection and destruction by the immune system. However, they often escape immune surveillance. This is for two major reasons: (i) tumor antigens are still self-antigens against which immune tolerance has been centrally established (Klein et al., 2009); and (ii) because cancerous tissue exploits several mechanisms to suppress anti-tumor immunity (Khong and Restifo, 2002). These include, but are not limited to, the downregulation of human leukocyte antigens (HLA) and thus diminishment of tumor antigen presentation, up-regulation of inhibitory co-stimulatory molecules, downregulation of receptors for cytolytic and apoptosis-inducing molecules such as Fas ligand and tumor-necrosis factor α (TNF-α), secretion of immunity inhibiting molecules, recruitment of regulatory T-cells (Treg) or induction of antigen presenting cells (APC) with tolerogenic potential (Khong and Restifo, 2002). All these factors contribute to the formation of an immunologically suppressive tumor microenvironment (Hanahan and Weinberg, 2011). Moreover, uncontrolled targeting of self-antigens often results in the development of autoimmunity and on-target/of-tumor toxicity (Klebanoff et al., 2016; Koneru et al., 2015; Lamers et al., 2006).

For the above reasons, much effort has been, in recent years, invested into the development of alternative approaches for supplementing traditional therapy. The field of immunotherapy showed signs of robust advancement during the last decades (Kalos and June, 2013; Miller and Sadelain, 2015). Today, scientists and clinicians are able to induce specific immune responses against tumor by several non-specific as well as antigen-specific approaches (Hinrichs and Restifo, 2013; Miller and Sadelain, 2015). This advancement has been possible due to generation of several transgenic mouse models which play indispensable role in academic and clinical research, cancer therapy including. Furthermore, the development of monoclonal antibodies, methods of adoptive cell transfer and safe methods of cell-engineering via genetic

[1] http://apps.who.int/gho/data/view.wrapper.MGHEMORTCAUSE10-2012?lang=en&menu=hide
[2] http://www.who.int/features/factfiles/cancer/facts/en/

modification of different immune cell populations, for example with chimeric antigen receptors (CARs) (Sadelain et al., 2003; van der Stegen et al., 2015), allows so far unprecedented accuracy and efficiency in tumor targeting. Current immunotherapy, however, still lacks the capacity to fully control and fine-tune the outcome of these approaches. Further advancement in understanding how to control the specificity, location, timing and level of effector functions are the subject of current research in this field.

Genetic modification of T-cells by CARs is a revolutionary technology emerging in the field of tumor immunotherapy and relatively rapidly expanding its applications. CARs combine the antigen recognition domain of a monoclonal antibody with the signaling domains of a T cell receptor (TCR) and co-stimulatory molecules and endow T-cells with the capacity to recognize the antigen of interest in a MHC-independent manner (Maher, 2012). Since the first efforts to genetically modify T-cells for the purpose of cancer therapy in early 1990s, significant amount of money has been invested into this field primarily in the United States. To this date, several research centers harboring numerous CAR clinical trials have been established not only in the United States but also in Europe and Asia (Appendix - Table 15).

In this thesis I briefly summarize the current knowledge concerning immune responses to cancer and focus more specifically on the use of CAR expressing T-cells for immunotherapy of B-cell leukemias. I will continue with a brief overview of the recent and ongoing clinical trials exploring CAR technology and describe the current limits of its application for solid tumors. I will conclude the theoretical part with the most recent data concerning the advancement in fine tuning approaches for CAR applications.

The experimental part of this work was performed in cooperation with Dr. Pavel Otáhal, from the First Medical Faulty at Charles University in Prague and focuses on the development of functional CAR constructs which could be potentially used for T-cell mediated therapy of B-cell leukemia in local clinical environment. Specifically, I focused on the preparation of CAR constructs targeting B-cell specific surface markers CD19 and CD20, as well as on CAR construct with dual specificity, so called superCAR, which would encode for CARs simultaneously targeting both CD19 and CD20. The activation capacity of all three CAR-T-cell systems will be tested and compared. In the discussion, I will touch upon the most important conclusions from this study which are put into context with other ongoing worldwide research efforts and highlight possible future directions in this rapidly developing field of translational research.

2. Overview of literature

2.1. Immune system versus cancer

Cancer is a very heterogeneous group of diseases that can effect practically any tissue. They all have in common progressive growth of a single transformed cell that has escaped the immune system surveillance (Hanahan and Weinberg, 2011). Approximately 14 million of new cases of cancer appear each year and this number is expected to rise in upcoming years[3]. In 2012, the most frequently diagnosed were lung, prostate, colorectum, stomach and liver cancer among men and breast, colorectum, lung, cervix and stomach cancer among women[4].

In the 1950s, when inbred mice strains became available, the study of immune responses to tumors became experimentally more accessible. In 1957, the idea that tumors are immunologically distinct and that the immune system can recognize and destroy cancerous cells was proposed by Macfarlane Burnet and Lewis Thomas as the immunosurveillance hypothesis (Burnet, 1970). They positioned lymphocytes into the center of immune responses whereby they recognize transformed cells. Experiments that followed focused on the very basic question, i.e. whether immune system is necessary to recognize and suppressed tumor growth. The results from these initial experiments did not show much of supporting evidence for the immunosurveillance hypothesis. Notably, athymic nude mice (Flanagan, 1966; Pantelouris, 1968) did not form chemically-induced tumors at higher frequency than wild type mice and thus this hypothesis became abandoned (Stutman, 1974). Later on, it became clear that the experiments had several drawbacks. First, nude mice are not completely immunocompromised, but contained a detectable population of αβ T-lymphocytes (Maleckar and Sherman, 1987). Second, at that time it was also not known that some inbred strains are less sensitive to tumor-inducing chemicals due to distinct composition of their bio-converting enzymes. Third, the monitoring periods may also have not been long enough to support the development of some types of tumors. Forth, important cell populations, such as γδ T-lymfocytes and NK cells, have not yet been characterized at that time. And fifth, the lack of a mouse strain that would have a mutation specifically affecting critical immune tissues and cell lineages, prevented the design and performance of crucial causal experiments (Dunn et al., 2002).

Renaissance of the cancer immunosurveillance hypothesis arouse in the 1990s when Dighe et al. showed that endogenously produced interferon (IFN) γ protected the host from cancer growth

[3] http://www.who.int/mediacentre/factsheets/fs297/en/
[4] http://gco.iarc.fr/today/home

(Dighe et al., 1994). Another important discovery was that the component of granules of cytolytic T-lymphocytes and natural killer (NK) cells – perforin – is essential for lymphocyte-dependent killing of tumor cells. Perforin-deficient mice developed more chemically induced tumors than perforin-sufficient mice treated in the same way (van den Broek et al., 1996). It thus became clear that components of the immune system significantly influence tumor development. Critical experiments were conducted when mice lacking the recombination activation gene (RAG) 1 and (RAG-2) were generated. These mice are unable to rearrange lymphocyte antigen receptors and thus lack T-cells, B-cells, NK cells and NK T-cells (Shinkai et al., 1992). When experiments with chemically-induced tumors were performed on RAG knock-out mice, they developed tumors more rapidly than wild-type mice (Shankaran et al., 2001). Disruption of other components of the immune system revealed that both the innate and adaptive arms of immune system are involved in cancer immunosurveillance (Dunn et al., 2002). In humans, the cancer immunosurveillance hypothesis is further strongly supported by the presence of tumor infiltrating lymphocytes (TILs) in some patients, whose presence is associated with better prognosis (Figure 1a) (Clemente et al., 1996). However, the positive impact of TIL is ambiguous. A substantial part of the lymphocytes present in the tumor microenvironment may be represented by Tregs which have a suppressive potential (Pichler et al., 2016).

Nevertheless, even a fully competent immune system is not "almighty" and malignant transformed cells may escape immunosurveillance Figure 1c). As mentioned above, the role of the immune systems is to protect its host and maintain homeostasis, but it doesn't stop here. It has been demonstrated that it also has the potential to shape tumor development. Tumors that form in the absence of the immune system tend to be less immunogenic than those that develop in its presence (Shankaran et al., 2001). Therefore a more accurate term "cancer immunoediting" has been proposed by Dunn and colleagues (Dunn et al., 2002) and this feature is now considered as one of the hallmarks of cancerous tissues (Hanahan and Weinberg, 2011). The authors described three successive phases characterizing ever-evolving relationship between the host and tumor, which they designated as the three "Es" of cancer immunoediting (Figure 1). The first phase termed as "elimination" is a period when cancer immunosurveillance is very efficient and dominates. Transformed cells are recognized in time and eliminated (Figure 1a). In the second phase - termed "equilibrium", tumor variants accumulate and select mutations that increase their resistance to immunosurveillance, but are still kept in check by the immune system in a dynamic equilibrium (Figure 1b). If such equilibrium is disrupted, these resistant variants may "escape" and cause tumor growth and the manifestation of cancer, which

corresponds to the third phase of cancer immunoediting (Figure 1c) (Dunn et al., 2002; Khong and Restifo, 2002).

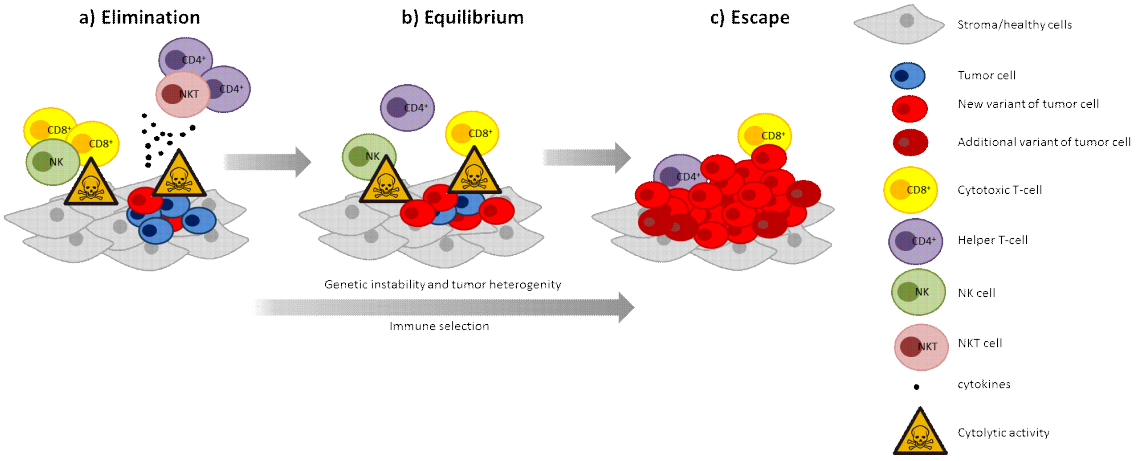

Figure 1: Cancer immunoediting *- figure is adapted from (Dunn et al., 2002).*

a) Malignant cells form constantly in the organisms, but during the elimination phase the immune system manages to recognize and destroy these potentially harmful cells. This period corresponds to immunosurveillance. b) Antitumor immunity creates selection pressure on cancer cells and the tumor accumulates beneficial mutations that give rise to more resistant tumor cell variants. The immune system does not manage to destroy all cancer cells but keeps them under control in a dynamic equilibrium. c) If the immune system is weakened and resistant tumor cells manage to escape immunosurveillance, they rapidly proliferate and tumor develops.

Today, we have a much more comprehensive understanding of the interplay between the host and the developing tumor. Tumor elimination is initiated by the innate immune responses which subsequently activates adaptive immunity. When transformed cells reach a critical mass, they begin to invade the surrounding tissue and promote angiogenesis (Hanahan and Weinberg, 2011) associated with inflammation. The increased blood flow allows the entrance of innate immune cells such as NK cells, NK T-cells, γδ T-cells, neutrophills, macrophages and dendritic cells that collectively recognize damage associated molecular patterns (DAMPs) present in the site of initial inflammation (Dunn et al., 2002). IFNγ production by innate immune lymphocytes enhances antitumor response not only by inducing cell death but mainly by promoting production of chemokines that support angiogenesis and attract cells of the adaptive immune system (Shankaran et al., 2001). Antigen specific $CD4^+$ and $CD8^+$ T-cells that have been primed by a specific tumor antigen, migrate to the draining lymph nodes, where they expand and mature into $CD4^+$ helper and $CD8^+$ effector cytotoxic T-lmphocytes. Cytotoxic

T-cells are the only antigen specific effector cells available for tumor eradication. They poses several cytolytic functions for killing antigen bearing tumor cells such as the ability to lyse target cells by secretion of perforines and granzymes or by FasL-mediated apoptosis (Sadelain et al., 2003).

At the same time however, effective antitumor immune response creates a selective pressure on tumor cells. These cells are genetically very unstable and accumulate large numbers of mutations. Those variants that accumulated the most resistance-mediated mutations, render the tumor less visible for immune system detection and more aggressive to overcome established dynamic equilibrium and escape immunosurveillance (Dunn et al., 2002; Khong and Restifo, 2002). Thus, natural selection pressure exerted by the host immune system generates resistant tumor variants that have acquired several escape attributes. These are similar to those used by microorganisms to evade immune responses and are highlighted in Table 1 (Khong and Restifo, 2002; Muenst et al., 2016).

Hematologic malignancies possess several noticeable characteristics. Majority of blood tumors are diffused and therefore compact mass does not represent an obstacle for the initiation of immune responses as it is with solid tumors (Bachireddy et al., 2015). Another advantage is their close proximity to the cells of the immune system which renders hematologic malignancies relatively immunologically responsive (Del Giudice et al., 2009; Savarrio et al., 1999). Blood malignancies are often a consequence of prior therapy but majority of cases manifest *de novo* (Sill et al., 2011). They frequently possess chromosomal aberrations. Gain-of-function mutations in components of signaling pathways lead for example to constitutive signaling in the IL-7 pathway in T-cell acute lymphoblastic leukemia (ALL) (Ribeiro et al., 2013) or to constitutive signaling in the absence of antigen through the B-cell receptor in diffuse large B-cell lymphoma (Davis et al., 2010). The pathology of hematological malignancies therefore manifests by abnormal proliferation and differentiation of a single lymphoid or myeloid cell clone and this neoplasm then hijacks the immune system and limits or alters its physiological functions (Bachireddy et al., 2015). Unfortunately gain-of-function mutations in components of signaling cascades result in the absence of specific tumor antigens. However an important feature of blood malignancies is that lymphoid and myeloid cells possess lineage and differentiation state-specific markers that are characteristic for a certain blood subpopulation and may be used for antigen specific activation of cytotoxic lymphocytes. Lineage markers and the fact that cells are diffused also facilitates their isolation and these malignancies are therefore well studied and therapy in this field is developing rapidly (Bachireddy et al., 2015).

Evasion attribute	Outcome
Genomic instability	generation of a large repertoair of subclones that are selected
Downregulation/loss of MHC I	downregulation of antigen presentation
Loss of tumor antigens	reduced visibility for tumor specific T-cells
Lack of costimulation	induction of anergy in antigen specific T-cells
Impaired death receptor signaling pathway	resistance to apoptosis
Overexpression of serin proteas inhibitor	block of cytotoxic activity of CTL
Secretion of immunosupressive cytokines and chemokines	suppression of inflammation, generation of tumor microenvironment
Activation of T-cells	activation induced cell death
Role of regulatory T-cells	suppressive environment

Table 1: Immune evasion mechanisms involved in tumor escape.

Tumor cells are genetically very unstable and natural selection favors tumor cells that have acquired immune evasion attributes. Tumor cells hide from the immune system by downregulating the expression of MHC class I molecules and therefore reduce their antigen presentation. Independently of MHC class I antigen presentation, tumor cells also lose tumor antigens to reduce their visibility for tumor specific T-cells. In the tumor microenvironment, inflammation may be suppressed by secretion of immunosuppressive growth factors and cytokines such as vascular endothelial growth factor (VEGF), interleukine (IL) 10 and transforming growth factor β (TGF-β). This results in the lack of co-stimulatory molecules on tumor cells and their recognition by tumor specific T-cells results in T-cell anergy or conversion to Tregs. Tumor cells often downregulate or acquire mutation in the signaling cascade of death receptors such as Fas or tumor necrosis factor-related apoptosis inducing ligand (TRAIL). Downregulation or loss of these molecules renders tumors resistant to Fas-FasL activation induced cell death (AICD) and TRAIL induced apoptosis. Negative feedback loop in T-cell activation may be misused for tumor evasion. Antigen recognition by tumor specific T-cells induces upregulation of the death receptor ligand FasL causing apoptosis of these activated cells but also of other surrounding lymphocytes bearing Fas receptor. Tumor cells may also actively block the cytotoxic capacity of cytotoxic lymphocytes by upregulation the protease inhibitor 6 which causes the inactivation of the effector molecule granzym B .(Khong and Restifo, 2002; Muenst et al., 2016)

Above I have briefly summarized the basic features surrounding tumor immune responses and the mechanisms of tumor escape. To better understand the underlying idea surrounding

the development of CARs, how they function and to better appreciate their benefits in cancer immunotherapy, in the next section I will briefly describe the nature of classical T-cell receptor signaling cascade and its effector functions.

2.2. Classical T-cell signaling pathway

Cell signaling is most studied among T-cells. Their signaling represents a very complex and meticulously assembled network of a large number of proteins that together transduce the extracellular engagement of TCR with peptide-MHC (pMHC) across the plasma membrane (PM) and generate an intracellular signal which initiates the process of T cell activation. The TCR and signaling proteins are spatially integrated into signaling modules, whose function is to generate, modify and convey the signal in a way that the T cell can fulfill its signaling-related effector functions. Acuto and colleagues, formally defined three major and distinct TCR modules: (i) the TCR triggering module; (ii) the regulation module and (iii) the signal diversification and regulation module (Acuto et al., 2008) which are depicted and described in Figure 2. In general, this network of signaling cascades leads to the activation of three critically important signaling pathways which activate transcription factors nuclear factor kappa B (NF-κB), nuclear factor of activated T-cells (NFAT) and activator-protein 1 (AP-1) which, in turn, mediate cytokine production, cell proliferation and T cell differentiation (Malissen and Bongrand, 2015). Besides robust changes in gene expression profile, the activation through the TCR also leads to the regulation of cell adhesion and cytoskeleton rearrangement (Acuto et al., 2008; Brownlie and Zamoyska, 2013) (Figure 2).

The below described complexity of TCR signaling and its variegated outcomes allows T-lymphocytes to be involved in and to regulate a plethora of various physiological events. This concerns the generation of T cells in the thymus, physiological roles of T cells in the periphery and differentiation of $CD4^+$ T-cells into various subclasses of helper cells, such as Th1, Th2, Th17, to mention just those most studied. In the thymus, the outcome of TCR signaling is essential for appropriate positive and negative selection of thymocytes whereby harmful autoreactive T-cell are removed and $CD4^+$, $CD8^+$, Tregs and other types of T cells with a diverse repertoire of TCR are generated. In all of these processes, it seem that the strength of antigen recognition by TCR determines the functional outcomes (Acuto et al., 2008; Filipp et al., 2012; Klein et al., 2009).

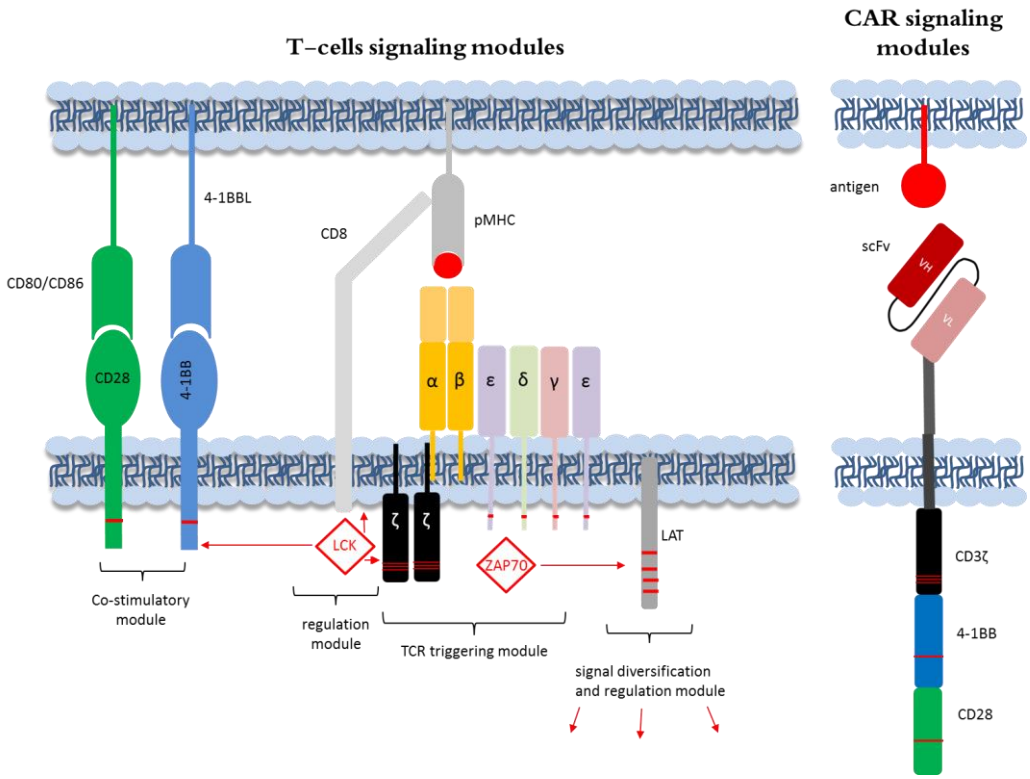

Figure 2: TCR signaling complex

The TCR signaling machinery can be divided into three modules. The TCR is represented by the αβ heterodimer which associates with CD3εδ, CD3εγ and CD3ζζ. Each ε, γ, and δ polypeptide contain a single immuunoreceptor tyrosine activation motif (ITAM) in their cytoplasmic domain (red line). The ζ polypeptide has three ITAMs in its cytoplasmic domain. Upon antigen recognition, the co-receptor CD8 brings the protein lymphocytes specific tyrosine kinase (LCK) into the proximity of CD3 ITAMs that then become phosphorylated. This leads to the recruitment of ζ-chain associated protein kinase of 70kDa (ZAP-70) which in turn phosphorylates the tyrosine residues of the scaffold protein linker for activation of T-cells (LAT), and the Src-Homology 2(SH2) domain-containing lymphocyte protein of 76kDa (SLP76) (not shown). SLP76 then recruits different proteins essential for signal diversification and leads to three important signaling pathways. SLP76 activates phospholipase C-γ (PLCγ) which generates signaling molecules diacylglycerol (DAG) and inositol triphosphate (IP_3). The signaling pathway involving DAG culminates in the activation of the transcription factor NFκB. The pathway involving IP_3 activates the transcription factor NFAT. SLP76 also phosphorylates GTP exchange factors (GEFs) which lead to the activation of a mitogen-activated protein (MAP) kinase cascade that activates the transcription factor AP-1. These three transcription factors induce specific gene expression leading to cytokine production such as the production of IL-2, cell proliferation and differentiation (Acuto et al., 2008; Brownlie and Zamoyska, 2013; Filipp et al., 2012; Malissen and Bongrand, 2015). For comparison, the CAR receptor and its TCR-derived modules is shown in corresponding colors.

In the context of this chapter, it is important to mention a very important caveat which must be considered when immune anti-tumor responses are studied. During T-cell development, the mechanisms of central tolerance in the thymus delete self-reactive T-cells or convert them to Tregs (Anderson et al., 2005; Liston et al., 2003). Self-reactive T-cells are selected during negative selection through the interaction with medullary thymic epithelila cells (mTECs) which ectopically express tissue specific antigens, sizeable portion of them under the control of autoimmune regulator, AIRE (Derbinski et al., 2001). This mechanism provides protection against autoimmunity, however it also challenges anti-tumor immunity because it eliminates potential tumor-specific T-cells (Kyewski and Klein, 2006). Tissue specific antigens expressed in the thymus may also be expressed on tumors as TSAs. However, due to negative selection mechanisms high affinity $CD8^+$ T-cells are deleted and medium affinity $CD4^+$ T-cells are converted to Tregs (Klein et al., 2014). Therefore, what is left of the tumor-specific T-cell repertoire are low affinity T-cells that are not sufficient to potentiate satisfactory anti-tumor immunity (Bos et al., 2005). Blocking central tolerance through the deletion of mTECs is one of the many emerging ways of enhancing anti-tumor immunity (Khan et al., 2014)

Essential for optimal helper T-cell activation and therefor also for the generation of a potent anti-tumor immune response is not only the signaling through pMHC-TCR interaction, termed as signal 1, but also signaling through distinct co-stimulatory receptors presented on T-cells and APCs, termed as signal 2 (Lafferty and Woolnough, 1977). In the absence of co-stimulation, T-cells recognizing pMHC fail to get activated, and get depleted or enter the unresponsive state of anergy (Schwartz, 1990).

The fundamental co-stimulatory molecule is CD28 (Aruffo and Seed, 1987). It provides an obligatory second signal needed for the development of immune response to an antigen (Lafferty and Woolnough, 1977). CD28 is a disulfide-linked membrane homodimer receptor with extracellular immunoglobulin (Ig) -like domains essential for the secretion of IL-2, expression of IL-2 receptor and cell-cycle progression (Harding et al., 1992; Jenkins et al., 1991; June et al., 1987). The receptor binds structurally homologous ligands B7.1 (CD80) (Freedman et al., 1987) and B7.2 (CD86) (Freeman et al., 1993) expressed on professional APCs. The second member of the CD28 family is cytotoxic T- lymphocyte antigen 4 (CTLA-4) which counteracts CD28 signaling. Its expression is induced upon T-cell activation and delivers a negative signal attenuating T-cell activation (Walunas et al., 1994).

Additional co-stimulation molecules have been identified. The induced co-stimulator (ICOS) belongs to the CD28 family and binds ICOS ligand. ICOS is expressed *de novo,* unlike the constitutively expressed CD28, and its interaction with-ICOS ligand induces production

of IFN-γ, IL-2, IL-4 and IL-10 (Coyle et al., 2000; Hutloff et al., 1999). Targeting these co-stimulatory molecules has shown to be a perspective therapeutic approach and this strategy became a sub-field of immunotherapy (Linsley and Nadler, 2009).

Structurally different co-stimulation molecules are members of the tumor necrosis receptor family (TNRF) and are equally important for the generation of many types of T-cell responses (Chen and Flies, 2013; Croft, 2003). Receptors such as 4-1BB (Kwon and Weissman, 1989), also known as CD137, OX-40 (Paterson et al., 1987), known as CD134 or TNFRSF9, or CD27 (van Lier et al., 1987), known as TNFRSF7, are cell membrane molecules distinctly upregulated on activated T-cells. They provide the late-activation signal and promotes T-cell proliferation, cytokine production and survival (Croft, 2009). Based on these characteristics, these co-stimulatory receptors are being studied for potential therapeutic utility as well (Ascierto et al., 2010).

It has been shown that when antigen load is high, as is the case during viral infection or within the tumor microenvironment, antigen specific T-cells get exhausted, lose their effector functions and are deleted (Virgin et al., 2009; Wherry et al., 2003; Zajac et al., 1998). Exhaustion has been most studied in $CD8^+$ cytotoxic T-cell, however it seems that $CD4^+$ T-cells follow similar principles and can as well acquire exhaustion phenotype (Antoine et al., 2012). This distinct stage of terminal T-cell differentiation is characteristic by upregulation of co-inhibitory molecules such as CTLA-4 or programed cell death protein (PD-1). PD-1 and its ligand are probably the most prominent inhibitory molecule associated with T-cell exhaustion (Wherry, 2011). These molecules are expressed to some extent on functional effector T-cells as part of a negative feedback loop, however long-lasting or high expression of antigen manifests in T-cell exhaustion (Virgin et al., 2009). Both CTLA-4 and PD-1 inhibit T-cell signaling. PD-1 ligand (APC) binds PD-1 (T-cells) and recruits several phosphatases to the proximity of the TCR which attenuates signaling (Parry et al., 2005). CTLA-4 on the other hand competes with CD80 and CD86 for CD28 (Pentcheva-Hoang et al., 2004). T-cell exhaustion occurs in a hierarchical manner - they gradually lose their ability to produce IL-2, to proliferate and to kill *ex vivo* as well as their capacity to produce TNF, IFN-γ and to degranulate. The final exhaustion stage is the loss of antigen specific T-cells (Wherry et al., 2003; Zajac et al., 1998).

Blocking the PD-1 or CTLA-4 pathway is one of many emerging therapeutical strategies for controlling cancer immunosurveillance (Brahmer et al., 2010). Interestingly, it has been showed that T-cell exhaustion can be rescued by co-stimulation. The application of a 4-1BB specific mAb together with IL-7 restored $CD8^+$ T-cell effector functions (Wang et al., 2012).

Ligands to the above mentioned co-stimulatory and co-inhibitory molecules are expressed primarily by APCs what make these cells a potential target for manipulation in the context of immunotherapy. However the expression of these molecules is not exclusive and T-cell activation may be therefore influence by other immune and non-immune cells (Croft, 2009).

This capacity to communicate with the environment via a plethora of distinct surface receptors, places T-cells among the most important cells in the adaptive immune system. In spite of this seemingly overwhelming complexity including MHC restriction, $CD4^+$ and $CD8^+$ co-receptor signaling, co-stimulatory molecules, cytokine milieu, type of APC etc., some data points to the fact that much simpler schematics of TCR signaling are also functional (Irving and Weiss, 1991). Moreover, there is a striking difference between $CD4^+$ and $CD8^+$ cells in their ability to differentiate into various subtypes (Luckheeram et al., 2012). While $CD4^+$ differentiate into several T helper subclasses, $CD8^+$ cytotoxic T cell's main function is to kill target cells. So far, the ability to differentiate into various subtypes of $CD8^+$ T cells has not been reported. Thus, while the signaling in $CD4^+$ T cells might be quite complex in physiological scenarios in order to ensure the fine tuning of their responses and differentiation program, such complexity might not be required for the on/off activation of cytotoxic function in $CD8^+$ T cells via their TCR. Recognition of this fact led to the development of several distinct approaches which aim to unlock the killing potential of $CD8^+$ T cells or NK cells and use them to target and kill tumors (Eshhar et al., 1993; Gross et al., 1989). There are several experiments which lend credit to this idea. These experiments are described in the next section.

2.3. Chimeric antigen receptors (CARs)

Immune responses to cancer are mediated by main effector cells of the immune system – $CD8^+$ T-lymphocytes (Barry and Bleackley, 2002). The tumor-killing potential of these cells, which is rather low due to selection in the thymus, is unfortunately further significantly suppressed in cancer patients. For this reason, adoptive transfer of genetically modified T-cells has tremendous potential in the field of immunotherapy. With advances in cell cultures and gene engineering it has become possible to redirect T-cells specificity with TCR-transfer (Stanislawski et al., 2001) or with chimeric TCR (Srivastava and Riddell, 2015). One of the most perspective immunotherapeutic approaches developing in the past decades is the adoptive transfer of chimeric antigen receptor engineered T-cells (June et al., 2015).

In 1989 the group of Z. Eshhar published the study dealing with the possibility of constructing a synthetic chimeric TCR where its extracellular portion is replaced with antibody-derived specific antigen recognition domain (Figure 3) (Gross et al., 1989). In 1993 the same group discovered that the CD3ζ signaling domain alone is sufficient to trigger a TCR signaling

cascade and they constructed the first generation of CARs (Figure 3) (Eshhar et al., 1993). These antigen receptors share many attributes with conventional TCR but also show a number of fundamental differences (Table 2). CARs are artificial constructs consisting of a target protein-recognizing antibody fragment and signaling domain(s) derived from the TCR complex. Their structure is described in more detail in Figure 3. It is important to emphasize that to ensure selective anti-tumor responses, the target sequence recognized by antibody fragment must be practically exclusively expressed on tumor cells. Constructs encoding these chimeric receptors are *in vitro* transfected into isolated patient´s T-cells. These manipulated T-cells are then adoptively transferred back to patients where they recognize tumor-derived target protein and initiate the killing of tumor tissue (Sadelain et al., 2003).

2.3.1. Structure and signaling

CARs are fusion molecules composed of an extracellular antigen recognition domain, typically derived from the single chain fragment variable (scFv) of a monoclonal antibody (mAb), a flexible spacer region, a transmembrane spanning sequence and an intracellular CD3ζ signaling domains derived from the TCR associated signaling CD3 complex (Figure 3) (Heiblig et al., 2015; Jensen and Riddell, 2015; Maher, 2012).

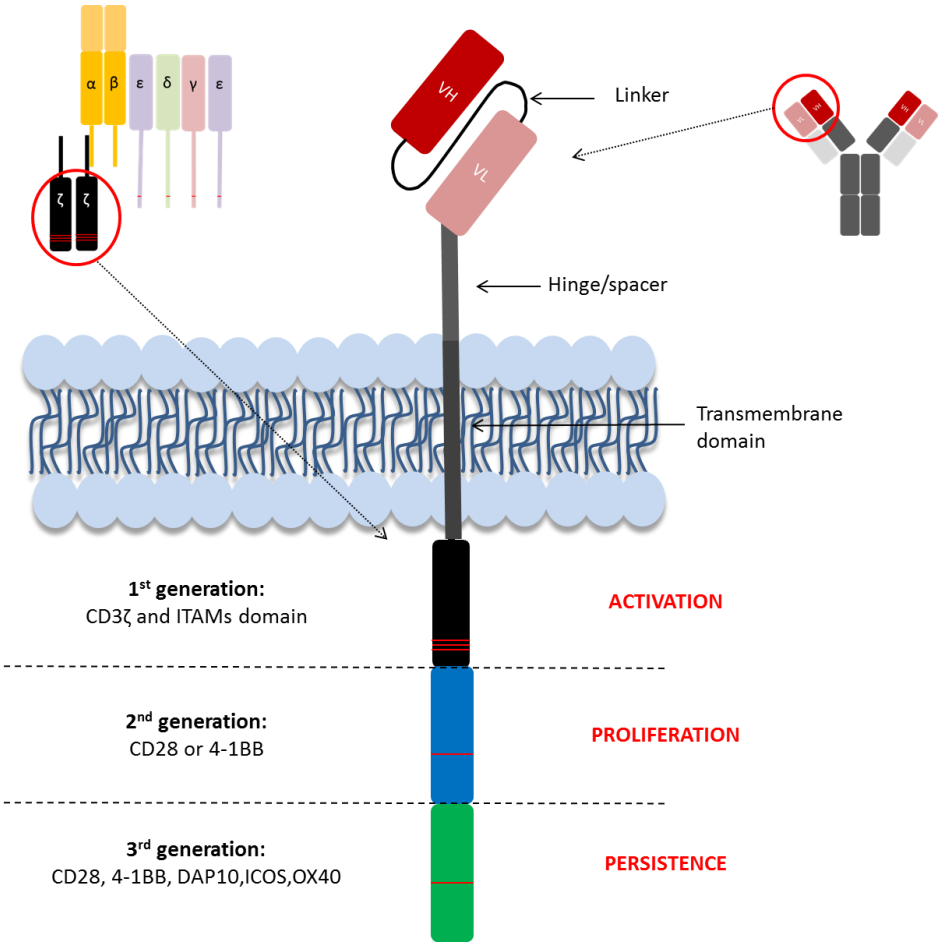

Figure 3: Chimeric antigen receptor structure.

The extracellular antigen recognition domain is composed of the variable light (VL) and heavy (VH) chains derived from an immunoglobulin molecule specific for an antigen of interest. The extracellular and transmembrane domains are connected by a flexible spacer sequence often derived from CD8 or CD28 subunits. The transmembrane domain is associated with the CD3ζ activation domains carrying ITAMs by electrostatic interactions. In second generation CARs the activation domain is further attached to the signaling sequence derived from the co-stimulatory molecules most often CD28 or 4-1BB which improves CAR T-cell proliferation. The cytoplasmic part of third generation CARs is further supplemented with additional one or more sequences from co-stimulation molecules that enhance CAR T-cell persistence. Inspired by (Heiblig et al., 2015).

CAR antigen recognition is mediated by the scFv and unlike the recognition by αβ TCR, CARs function independently of MHC. Therefore their application is limited to the recognition of cell surface antigens which are available for interaction with the CAR. On the other hand, the use of a scFv renders the CAR T-cell independent of peptide processing and presentation by the target cell (Maher, 2012). This process is often disrupted in cancer cells (Dunn et al., 2002). Antigen recognition is therefore not limited to peptides but is endowed to other molecules like glycolipids and carbohydrates as well (Sadelain et al., 2015). Because of the MHC alloreactivity, the MHC independent antigen recognition makes CARs potentially applicable to all patients bearing the same tumor antigen. Antigen recognition by CAR T-cells also differs in affinity and sensitivity compared to traditional TCR. Because tumor specific T-cell, if present, are mostly low affinity, CARs bind antigens with higher affinity (Harris and Kranz, 2015). However TCR are more sensitive in antigen recognition than CARs. For T-cell activation it is theoretically sufficient to have one antigen molecule on the target cell surface (Sykulev et al., 1996). On the other hand, CAR T-cells are activated by cross-linking, so they require in minimum a 100x higher antigen concentration on target cells (Harris and Kranz, 2015) (Table 2). Higher affinity to antigen does not necessarily result in higher activation of T-cells. In contrast low affinity immunoreceptors are more potent in discriminating target cells with low and high expression of antigen. Most tumor antigens are self-antigens that only differ in their expression level, time or location compared to physiological expression, so this must be taken into account when applying CAR T-cells to patients (Chmielewski et al., 2004). The design of the most convenient antigen recognition domain is further complicated by target epitope heterogeneity. For example, the antigen mucin 1 which is highly expressed in ovarian and breast cancer, is highly glycosylated and has different epitopes recognizable with distinct efficiency by CAR T-cells (Wilkie et al., 2008).

Following the antigen-recognizing scFv fragment of CAR is a space/hinge region which separates the extracellular antigen recognition domain from the membrane crossing sequence. This provides a certain range of flexibility to the receptor. Unlike conventional TCR where the antigen recognition is determined by the pMHC-TCR interaction, for CAR T-cells the antigen recognition is determined by the structure, level of expression and type of epitope (Hudecek et al., 2015). The spacer provides flexibility and the ability to "reach" the target antigen (Guest et al., 2005). The ideal length of the hinge region is dependent on the dimension of the targeted surface antigen. The most common spacers used in CAR construction are derived from CD8α (short spacer), or from the IgG hinge (long spacer), typically IgG1 or IgG4, or from the Fc domain of IgG. When Fc derived domain is used, it must be altered or mutated to avoid *in vivo* interaction with physiological FcγR. Membrane proximal epitopes require

longer spacer domains for efficient T-cell activation (Harris and Kranz, 2015). In the case of CD19 epitope, a wide variety of spacers may be used *in vitro*, ranging from 12-229 amino acids. However, *in vivo* shorter spacers (12 aa) have proven to be more effective than intermediate (119 aa) or long spacers (229 aa) (Hudecek et al., 2015). It is therefore critical to consider the structure of the spacer domain based on the target when designing the optimal CAR because this domain dramatically affects *in vivo* antitumor activity.

The following transmembrane domain is structurally very important and affects CAR antigen recognition and signaling. This region is often covered by fragments derived from T-cell molecules like co-receptors CD3, CD4 or CD8, or co-stimulatory molecules CD28 or CD134 (also known as OX-40). The transmembrane domain derived from CD3ζ proved to be capable of heterodimerizing with the endogenous CD3ζ which leads to the upregulation of CD3ε and enhanced responsiveness to stimulation via CAR (Bridgeman et al., 2010).

The intracellular signaling region has been the most studied and subjected to various modifications throughout the years of CAR development. For signal transduction, the CD3ζ-derived signaling domain containing ITAMs or γ chain-derived fragment from the high affinity receptor for IgE – FcεR1 are routinely used. The first generation of CARs was very simple and signals provided by these receptors mimicked those provided by the natural TCR. These receptors had only the signaling domain in their cytoplasmic part (Figure 3) (Eshhar et al., 1993). Logically, the signaling outcome of this first CAR generation which lacked the capacity to generate co-stimulatory signals, was not the same as the outcome observed upon the activation of natural TCR. Therefore this domain became intensely modified and redesigned (Irving and Weiss, 1991). In physiological conditions, T-cell stimulation needs a second signal which is provided mainly by the co-stimulatory receptor CD28 (Geiger et al., 1999; Maher et al., 2002). The absence of such signal results in decreased proliferation of T-cells, T-cell anergy or their elimination by programmed cell death. In agreement with this notion is the fact that initial CAR construct that incorporated only the CD3ζ signaling domain failed to show clinically relevant success (Harding et al., 1992).

To overcome this drawback, the CD3ζ has been fused with a core segment of the signaling domain derived from a single co-stimulatory molecule, such as CD28 (Maher et al., 2002), 4-1BB (Long et al., 2015), CD27 (Song et al., 2012), ICOS (Guedan et al., 2014) or OX-40 (Finney et al., 2004). The co-stimulatory domains increased the effect of the signaling domain by mimicking the co-stimulation provided by APCs during physiological TCR recognition. A CAR fusion molecule providing the first and second signal together, was first constructed by Helene M. Finney and her colleagues (Finney et al., 1998). Co-stimulatory domain of CD28

and 4-1BB are most intensively studied and used as signaling domains in these CARs. The second generation of CARs enhances effector functions and T-cell proliferation, act on cell differentiation and cell death by increasing the expression of corresponding downstream regulators (Maher et al., 2002). It has been shown that the type of co-stimulatory signaling domain incorporated in the CAR molecule has significant impact on T-cell memory development, CAR signaling and CAR T-cell metabolism (Kawalekar et al., 2016).

CD3ζ-CD28 CARs showed enhanced T-cell proliferation *in vitro* and enhanced tumor killing *in vivo* (Finney et al., 2004). In addition, upon antigen encounter and receptor cross-linking, this construct effectively induces the secretion of IL-2 and cell proliferation which are features necessary for effector T-cell function and the induction of long-term memory (Maher et al., 2002; Zhong et al., 2010). Enhanced secretion of other cytokines such as IFN-γ, TNF-α and GM-CSF was also observed (Finney et al., 2004). When compared with 4-1BB containing CARs, CD28-CARs eradicate tumors at earlier time points (Zhao et al., 2015). However, their proliferative potential and persistence is much lower than in the case of 4-1BB CAR T-cells due to more rapid expression of exhaustion markers such as CTLA-4 or PD-1 (Kawalekar et al., 2016). Thus, while the addition of CD28 signaling domain delays activation-induced cell death of T cells expressing CAR construct, upon its repeated re-stimulation, the effect of CD28 co-stimulation diminishes (Long et al., 2015). It has been shown that in contrast to CD28 CAR T-cells, 4-1BB CAR T-cells induce $CD8^+$ central memory and CAR T-cell persistence (Kawalekar et al., 2016).

In this context it necessary to highlight the fact that major limiting factors for efficacy of CAR therapy is CAR T-cell exhaustion and antigen-independent CAR T-cell activation. As this directly relates to CAR-mediated co-stimulatory signaling, several studies addressed the question how these signaling moieties contribute to these phenomena. Adrienne H. Long and her colleagues. demonstrated that CD28-CAR exhaustion is due to antigen independent CAR activation caused by CAR clustering. The incorporation of the 4-1BB domain partly suppresses the development of exhaustion, leads to the increase of cytokine production, improves anti-tumor effect *in vivo* and increases *in vivo* persistence of CAR T-cells. By comparing the transcription profile of CD28-CARs and 4-1BB-CARs the group of Crystal Mackall identified that 4-1BB CARs showed higher expression of genes encoding transcription factors associated with memory and, on the other hand, lower expression of genes encoding exhaustion-related transcription factors. They also identified three pathways that might contribute to the improved functionality of 4-1BB-CARs such as response to hypoxia, cellular metabolism and negative regulation of apoptosis (Long et al., 2015). First clinical trial

incorporating the signalling domain of 4-1BB was performed by a group from the University of Pensylvania and two out of three patients in this trial experienced complete remission of chronic lymphocytic leukemia (CLL). CAR19 T-cells persisted in circulation for several months, proliferated and CAR expression memory T-cells were detected (Kalos et al., 2011; Porter et al., 2011).

As both molecules – CD28 and 4-1BB provide at the same time general and specific benefits, it has been investigated whether the co-expression of the two molecules along with the CD3ζ would lead to the enhanced cytolytic activity (through CD28) and enhanced CAR T-cell persistence (through 4-1BB). The co-expression of 4-1BB and CD28-derived signaling modules enhanced its effect and led to the increased potency and persistence of CAR T-cells after antigen stimulation (Zhong et al., 2010). For this reason, combinatorial fusion of CD28 and 4-1BB constructs has been marked as the third generation of CARs. Several studies confirmed the positive effect of the 3rd generation of CARs in enhancing T-cell responses (Carpenito et al., 2009; Wang et al., 2007). Notably, T-cell survival and activation was truly enhanced when combining 4-1BB and CD28 signaling domains through the upregulation of several anti-apoptotic factors from the TNF family, such as B-cell lymphoma 2, and maintained immune memory (Imai et al., 2004; Long et al., 2015; Zhong et al., 2010). However, it is very likely that to a certain extend 4-1BB stimulation was mediated by endogenous bystander T-cells. Final conclusions are therefore difficult to make because different studies focused on different CAR constructs, used different cohort of patients (age, type of malignancies) and many studies were not supported by *in vivo* experiments. The latter are essential since *in vitro* results often do not correspond to final *in vivo* observations.

	TCR	CAR
Antigen recognition		
Antigen recognition domain	$V_\alpha V_\beta / V_\gamma V_\delta$	Fab V_L V_H
MHC restriction	yes	no
Antigen origine	intracellular and extracellular	cell surface
Antigen type	peptides	peptides, carbohydrates
Signaling Machinery		
Number of subunits in receptor complex	10	1
Number of ITAMS	10	3
Number of tyrosines as substrates	20	6
Coreceptor, co-stimulatory involvement	yes (CD4, CD8, CD28 ect.)	none known
Affinity for antigen	10^4-10^6 M^{-1}	10^6-10^9 M^{-1}
Number of surface receptors per T-cell	50 000	> 50 000 but varies
Minimum number of antigens required	1	> 100

Table 2: Comparison of the conventional T cell receptor and the artificial chimeric antigen receptor.

The conventional TCR recognizes antigen in the context of MHC molecules through its variable αβ or γδ chains. CAR recognition domain is derived from an antibody VL and VH. The antigen recognized by the TCR is a peptide either derived from endogenous proteins (presented on MHC class I) or from exogenous proteins (presented on MHC class II). CAR T-cells function in a MHC-independent manner and therefore their recognition is not limited to peptide antigens. The TCR signaling machinery is composed of αβ TCR and CD3 chains and co-receptor CD4 or CD8). In contrast, CARs have a single CD3ζ signaling domain usually fused with a single co-stimulatory signaling sequence. The affinity of the TCR is in the order of micromoles while CAR affinity is much higher – in nanomolar values. Theoretically, the recognition of one single antigen molecule is sufficient to trigger T-cell activation. In contrast more than a hundred CAR receptors must be crosslinked for CAR T-cell activation. Adapted from (Harris and Kranz, 2015)

2.4. Treatment of hematological cancers

Since the invention of CARs in the 1990s, these synthetic receptors have gone a long way and have finally reached clinical trials (Appendix - Table 15). Some are more successful, some less. The success of this therapeutic approach is so far highly dependent on the type of disease and the nature of the targeted antigen. As mentioned above, CARs are MHC-independent. Therefore, ideal target antigens are primarily cell surface molecules and, owing to a killing efficiency of $CD8^+$ T cells, the greatest potential for their application seems to be cancer immunotherapy. However, a considerable complication is that TAAs are usually expressed on both healthy as well as on malignant tissues. Hence, the selection of appropriate tumor target antigen is of critical importance, otherwise CAR application can lead to on-target/off-tumor toxicity which can cause serious or even lethal side effects (Hinrichs and Restifo, 2013).

So far the biggest success in clinical application of CARs has been achieved in treating hematological cancers, specifically B-cell malignancies. This is due to the expression of B-cell lineage specific marker – CD19 (LeBien and Tedder, 2008). CD19 is a surface molecule expressed exclusively on B-cell lineage from pro-B-cells to mature B cells. Hematopoietic stem cells and other tissues lack CD19. This makes CD19 a hot candidate for CAR targeting. Importantly, it is expressed on almost all B-cell malignancies, except 5% of undifferentiated immature B-cell lineage in ALL. Very convenient is also the fact that long-term B-cell depletion and reduced immunoglobulin levels in patient treated with CD19 specific CARs is well tolerated due to immunoglobulin supplementation (Heiblig et al., 2015).

Another cell surface molecule expressed preferentially by B-lymphocytes is CD20. It's a mature B-cell marker that functions as a Ca^{2+} channel and is also being tested for malignant B-cell targeting (LeBien and Tedder, 2008).

CAR-targeted CD19 clinical trials have shown robust and lasting tumor regression in most pediatric and adult patients with relapsed/refractory ALL and in some patients with CLL (Grupp et al., 2013; Kalos et al., 2011; Porter et al., 2011).

In order to be eligible for allogeneic hematopoietic stem cell transplantation (allo-HSCT), patients must achieve complete remission by prior chemotherapeutic treatment. However many patients fail to meet these conditions and never receive potential life-saving allo-HSCT. Thus, for those patients that still have minimal residual disease (MRD), their chance for complete recovery is diminished compared to MRD^- patients (Brentjens et al., 2013). Therefore, the introduction of CAR technology into clinics is acclaimed as a long awaited breakthrough in the treatment of hematological malignancies.

2.4.1. Isolation and manufacturing of CAR T-cells

In clinical practice, manufacturing CAR T-cells begins with leukapheresis and T-cells isolation. This is a process where the whole blood of a patient is removed from the body, certain cellular component is selected and the rest is reintroduced back into circulation. In the case of CAR therapy, T cells are separated from the blood, *ex vivo* expanded, transduced to express an appropriate chimeric antigen receptor and finally adoptively transferred back into the patient (Davila et al., 2014a).

Leukapheresis should be applied before lymphodepleting chemotherapy in order to obtain sufficient T-cell numbers. According to the Department of Health and Human Services, the physiological T-cell count is between 500-1 200 cells/mm^3. The absolute T-cell count below 200-300/mm^3 would thus probably lead to poor T-cell collection. Apheresis normally takes place under hematopoietic stem cell mobilizing agent free conditions, but this varies depending on patient, the type of mobilizing agent and the type of T-cell isolation. Anti-coagulants are mixed with the blood in order to prevent blood clotting. The number of apheresis cycles depends on the number of target cells present in blood and on the desired final number of isolated cells. The apheresis product varies depending on patient's health conditions and their previous therapy. $CD3^+CD45^+$ T-cells can represent from 2.29% up to 4.67% of total blood count (Kalos et al., 2011). After isolating a sufficient number, T-cells are activated and expanded *in vitro*. The most convenient and Food and Drug Administration (FDA) approved method for T-cell activation is their stimulation via anti-CD3/anti-CD28 mAb coated magnetic beads (Levine, 2015).

2.4.2. CAR gene delivery systems

The next step is T-cell transduction with the CAR transgene. Mammalian cells can be transduced by several vectors. The most frequently used is viral vector transduction due to its high efficiency of gene delivery but transposon system such as the *Sleeping beauty* can be used as well (Kebriaei et al., 2016) Since retroviruses can permanently incorporate their genetic information into the host genome, they have been studied and utilized as natural gene delivery system for permanent cell transduction (Sakuma et al., 2012)

The retroviral genome is a single-strand RNA that encodes three large open reading frames *gag*, *pol* and *env* which are bounded by two long terminal repeats (LTRs) at the 5´and 3´end. These are required for viral transcription, reverse transcription and integration. The *gag* gene encodes viral core proteins, the *pol* gene encodes a set of enzymes needed for viral replication and the *env* gene encodes the viral surface protein. For safer use these viral genes are split into

separate plasmids thus limiting the formation of retroviral competent particles. A packaging cell line, most frequently the human embryonic kidney (HEK) cell line 293T, is then transfected with these multiple plasmids along with a plasmid carrying the exogenous DNA encoding CAR construct. Viral particles produced by HEK293T cells are then used to transduce target cells. Within these cells, the viral RNA is transported to the nucleus, reverse transcribed into dsDNA and randomly and permanently incorporated into the host genome ensuring a long-term gene expression. Retroviruses preferentially integrate into replicatively active euchromatine and such integration can cause the deregulation of physiological gene expression. This issue was resolved by the use of self-inactivating (SIN) vectors which create deletions in the LTRs and thus prevents the reactivation of provirus production, but not the expression of CAR construct. Unfortunately, retroviruses are unable to infect non-dividing cells which is highly inconvenient because majority of human T-cells are quiescent or dividing only occasionally (Vannucci et al., 2013).

Thus other vectors have been studies for gene transfer especially those derived from the human immudeficiency virus (HIV). Lentiviruses are closely related to retrovirus but are more advanced. They require more regulatory genes that neutralize host cell defense and effect viral replication. They can infect non-dividing cells and unlike retroviruses, they don't target cellular promotors for their incorporation into the genome, so the risk of insertional mutagenesis and oncogenicity is minimal. Similar as for retroviral vectors, for safety reason, the viral genes were split into separate packaging constructs and LTRs were mutated by the SIN vector. Lentivirus vectors have been further modified to transduce even wider range of cells by replacing the *env* gene with the highly conserved vesicular stomatitis virus glycoprotein (VSV-G) which is used for cell entry (Sakuma et al., 2012; Vannucci et al., 2013). After transduction, cells must be tested for the presence of replication competent viruses. If negative, then they are expanded *in vitro*, typically in bioreactors, to obtain sufficient number of cells. Finally after up to 20 days of cell cultivation and expansion, CD3/CD28 mAb coated magnetic beads are removed, cells are harvested, washed, concentrated and cryopreserved until being applied to the patient (Kalos et al., 2011; Levine, 2015).

2.4.3. Clinical application

The clinical application can slightly differ across clinical trials, but in principal they are the same. Once the genetically modified CAR T-cells are prepared, they are ready for patient application. *In vivo* studies on mice, as well as clinical studies have shown that for efficient CD19 specific T-cell-mediated targeting, some form of foregoing therapy is indispensable. Patients treated with cyclophosphamide before T-cell infusion showed enhanced persistence

of modified T-cells despite the fact that they have been infused with a lower number of CAR T-cells compared to the control group not treated with cyclophosphamide (Davila et al., 2013) Chemotherapy may increase the potential of CAR T-cells by stressing tumor cells, that have not been depleted by chemotherapy and making them more sensitive to cytotoxic CAR T-cells. It may also increase the potential of engraftment and migration towards tumor cells (Porter et al., 2011). Therefore the clinical protocol begins with the application of lymphodepleting chemotherapy. CAR T-cells are then applied by intravenous infusion in a split-dose approach. The total number of infused CAR T-cells varies depending on clinical trial as well. In the clinical study reported by Brentjens and his colleagues, the number of infused $CAR19^+/CD3^+$ T-cells ranged from $0.4\text{-}3.2x10^9$ (Brentjens et al., 2011). In the study published by Kalos and his group the total number of infused cells ranged from $0.3\text{-}5x10^9$ (Kalos et al., 2011). Neither of these studies discriminated $CD4^+/CD8^+$ T-cells for infusion. However the predominant infused T-cell phenotype was $CD4^+$. In CLL patients the mean of $CD4^+$ T-cells was 88% and the average ratio of $CD4^+/CD8^+$ was 10.5. On the other hand, in ALL patients the mean of $CD4^+$ T cells was diminished to approximately 63%. Despite the domination of $CD4^+$ T-cells, the number of $FoxP3^+$ regulatory T-cells was minimal (Brentjens et al., 2011).

After T-cell infusion, the trafficking of tumor specific T-cells to the site of tumor is monitored and the ability to persist and proliferate as well as the ratio of modified T-cells *in vivo* is analyzed. Renier J. Brentjens and his group analyzed the trafficking of CAR T-cell by immunohistochemistry of autopsies and showed that tumor specific T-cells truly migrate to lymph nodes, liver and bone marrow. They detected CAR19 T-cells 44 hours after infusion but also 2 months later (Brentjens et al., 2011). Michael Kalos and colleagues, who performed the study with CAR19 construct incorporating the co-stimulatory signaling domain of 4-1BB, detected CAR T-cell persisting in the blood even six months later (Kalos et al., 2011). Based on the ratio of isolated CAR19 T-cells analysis from patients 5 weeks post infusion, $CD4^+$ and $CD8^+$ transfected T-cells were endowed with the equivalent ability to proliferate and persist *in vivo* (Brentjens et al., 2011). Unfortunately several negative side effects are associated with CAR19 T-cell activation and proliferation (Brentjens et al., 2013). These are described in more detail in the next section.

2.4.4. CAR T-cell therapy related toxic effect

Cytokine-release syndrome

The serum and bone marrow cytokine levels are monitored to provide evidence of CAR T-cell function. However, CAR treatment is often accompanied with the cytokine release syndrome which is the most common toxic side effect of CAR T-cell therapy (Namuduri and Brentjens,

2016). The massive production of pro-inflammatory cytokines may occur several days to weeks after infusion of CAR T-cells as a consequence of T-cell triggering, activation and proliferation, and if not managed properly, it can cause life threatening complications. The symptoms vary depending on the severity of cytokine overproduction. Common are high temperatures, chills, or myalgias[5] but may escalate to life-threatening vascular leak, hypotension, respiratory and renal failure, cytopenias[6] and coagulopathy. Among the measured parameters are primarily serum and bone marrow markers of systemic inflammation such as cytokines, chemokines and other biochemical parameters (Brentjens et al., 2013; Kalos et al., 2011)(Table 3).

Cytokines and receptors
IFNγ
IL-6
IL-8
IL-2 receptor α
IL-2 receptor β
Chemokines
macrophage inflammatory protein 1α
macrophage inflammatory protein 1β
monocyte chemotactic peptide-1
CXC chemokine ligand 9
CXC chemokine ligand 10
Biochemical parameters
C-reactive protien
ferritin
aminotransferase
serum D-dimer
alkaline phosphatase

Table 3: List of most the commonly evaluated systemic inflammation markers associated with CAR T-cell therapy.

In the clinical study reported by Michael Kalos and his group, they measured a panel of 30 cytokines, chemokines and other relevant soluble factors in their clinical study. The most

[5] Muscle pain
[6] Reduction in the number of blood cells

relevant changes were detected for IFNγ which increased three fold above the base line (Kalos et al., 2011).

It has been shown that the severity of cytokine release syndrome correlates with the severity of leukemia. In other words, the higher the tumor burden, the more sever cytokine release syndrome (Maude et al., 2014). Among other complications with similar toxic side effects are macrophage activation syndrome and tumor lysis syndrome.

These post-infusion complication are well manageable by the application of IL-6-receptor blocking antibody - tocilizumab, glucocorticoids, corticosteroids and TNF inhibitor - etanercept (Grupp et al., 2013). In several clinical studies all patients suffering from cytokine release syndrome were treated by the above mentioned procedures and all of them fully recovered and their laboratory results returned to physiological values (Grupp et al., 2013; Maude et al., 2014; Porter et al., 2011). Interestingly, patients treated with CAR19 T-cells incorporating the signaling domain from the co-stimulatory molecule 4-1BB, instead of CD28, as in trials mentioned above, did not suffer from cytokine release syndrome. Some patients had higher levels of IL-6, IFN-γ or IL-2R but their clinical conditions did not require anti-cytokine treatment and pro-inflammatory cytokine levels reverted to physiological values despite continued function of CAR T-cells (Kalos et al., 2011).

B-cell aplasia

The absence of $CD19^+$ B-lymphocytes is a severe but well manageable toxic side effect accompanying properly functioning CAR19 T-cells and their persistence *in vivo*. This effect is detectable in all patients for which the therapy was successful. Unlike previously mentioned toxic side-effects, B-cell aplasia is a long-term effect. The absence of B-cells and therefore the disruption of humoral immunity are corrected and compensated by the intravenous infusion of immunoglobulins to maintain their necessary levels. The administration of antibiotics is also the part of such prevention and compensatory measures (Maude et al., 2014).

Encephalophaty

Neurologic toxicity may also occur after CAR T-cell infusion. Symptoms are high temperatures during which patients may experience delirium, aphasia[7] and hallucinations. Symptoms usually resolve after 2-3 days and the medication is not required (Maude et al., 2014).

The clinical procedure described above refers to B-cell malignancy treatment with CD19 specific CAR T-cells in general. However B-cell malignancies are a heterogeneous group

[7] Loss of speach

of leukemia and the treatment as well as the patient´s responses may differ depending on the specific type of leukemia or lymphomas. The transduction methods, optimal T-cell subtype, cultivation conditions, type of co-stimulation, presence of conditioning therapy or other immune enhancers and the degree of tumor burden must be taken into account when tailoring CAR therapy to a particular patient (Sadelain, 2015).

2.4.5. Anti-CD19 clinical trials

Many clinical trials employing CAR technology have been initiated and conducted in the United States. In general, their number has grown dramatically since the year 2000 (Figure 4a). Among the most targeted antigens, CD19 is at the top of the list (Figure 4b). The first report of CD19 CAR T-cell clinical trial was published by Kochenderfer and colleagues from National Cancer Institute in 2010. A patient with follicular lymphoma was treated with autologous anti-CD19-CAR transduced T-cells. Impressive partial remission lasted 32 weeks. $CD19^{+}$ blood cells were absent from week 9 to week 39 post CAR T-cell infusion (Kochenderfer et al., 2010). This success in cancer immunotherapy launched a wave of clinical trials using anti-CD19 CAR T-cells. The first clinical study with second generation CAR T-cells containing 4-1BB co-stimulation included three patients with CLL. Two out of three patients showed complete remission (Kalos et al., 2011) which lasted at least till September 2015 So far, the most dramatic success was reported in patients with ALL (Brentjens et al., 2013; Grupp et al., 2013; Maude et al., 2014). Renier J. Brentjens and colleagues from the Memorial Sloan Kettering Cancer Center treated 5 relapsed ALL patients with autologous CD19-28-ζ specific CAR T-cells. All patients demonstrated complete remission and no MRD was detected (Brentjens et al., 2013). In another study where Marco L. Davila and colleagues treated 16 ALL patients with CAR transduced T-cells, complete response was achieved in 88% of them and patients became eligible for allo-HSCT (Davila et al., 2014b). Shannon L. Maude and her team. from the Children´s Hospital of Philadelphia reported 27/30 children and adult patients with ALL complete remission and 6 months post infusion 68% of patients had CAR T-cell persistence and 73% had B-cell aplasia (Maude et al., 2014). A summary of actively recruiting or ongoing clinical trials is presented in Appendix – Table 15.

Even though these results are very promising, CAR T-cell therapy is still at its infancy and suffers from many limitations that are yet to be overcome. For example, as mentioned earlier, CAR T-cell therapy is still associated with diverse life threatening adverse effects and spatiotemporal aspects of this therapy are still difficult to regulate. Moreover, at present time, it is non-applicable to other types of cancers, such as solid tumors.

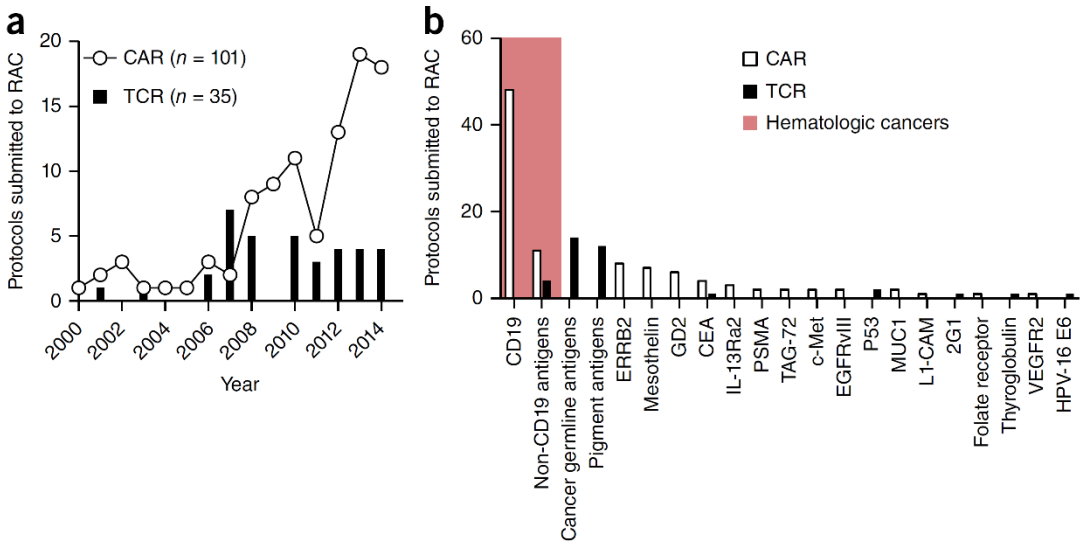

Figure 4: CAR and TCR clinical trials for oncology indications in the US between 1994 and 2014.

a) The total number of new CAR clinical trials for solid tumor immunotherapy throughout the years 2002 to 2014. b) The overall number of ongoing CAR clinical trials for tumor individual antigens. Reprinted by permission from Macmillan Publishers Ltd: Nature Medicine (Klebanoff et al., 2016), copyright 2016

2.5. Treatment of solid tumors

So far I have described the use of CAR T-cells only for B-cell leukemia which has shown to be the most effective due to the presence of the well-defined, B-cell lineage markers, CD19 and CD20.

However, solid tumor antigens have also been tested for the purpose of CAR T-cell therapy (Figure 4a and b). Treating solid tumors by CAR T-cells remains a challenge to this day. Even though the safety and feasibility of CAR T-cell treatment for certain solid tumors has been established in clinical trials, their application is still limited by off tumor/on target toxicity (Lamers et al., 2006). Very few antigens are uniquely specific for solid tumors. Target antigen candidates are most commonly "self" antigens that are co-expressed on healthy tissues which are therefore also attacked by CAR T-cells. Alternative way based on the targeting of neoantigens could decrease the level of autoimmune toxicity. Unfortunately, most solid tumors are in general not derived from constantly renewable tissues, like leukemia and lymphomas. Therefore, targeting a given tumor tissue as a single and oncoantigen specific entity, is not possible. The treatment of solid tumors using CAR T-cells is further complicated by the tumor microenvironment which actively suppresses the effector functions of T-cells. CAR T-cells need to traffic to the tumor site and actively invade to tumor mass in order to kill malignant cells. However, CAR T-cells cannot easily contact malignant cells because of the compact solid tumor architecture, local presence of inhibitory mechanisms and because of the possible absence of molecules on CAR T cells necessary for cell trafficking such as integrines, chemokines and chemokine receptors. These may be downregulated due to *in vitro* cultivation and genetic modifications of CAR T-cells (Guo et al., 2016).

An example of a targeted solid tumor antigen against which CAR T-cells are undergoing clinical trials is mesothelin. Mesothelin is a glycosylphosphatidyl inositol membrane-anchored glycoprotein. Its high expression was detected in many malignancies such as pleural and peritoneal mesothelioma or pancreas, lung, breast, ovarian and esophagela cancers. However mesothelin is also expressed on other tissues such as cornea, pleura, pericardium, peritoneum, tonsils, fallopian tubes or cervix. Because of heighten possibility for on target/off tumor toxicity, the FDA ordered very strict and low dosage phase I clinical study which is currently ongoing. Although it is too early to make any conclusions, so far the results have shown no on target/off tumor toxicity, but neither tumor regression (Klebanoff et al., 2016).

MUC16 is another candidate antigen expressed in the majority of ovarian cancers. It is a cell surface glycosylated mucin. Unfortunately, it is also expressed on other tissues, such as the eye.

Analogous to mesothelin, MUC16-specific CARs are in the “fine-tuning” dosage test in phase I clinical trials (Koneru et al., 2015).

CAR T-cells targeting another potential marker of solid tumors, carbonic anhydrase IX (CAIX), have already showed off tumor/on target toxicity in one clinical study. CAIX is expressed on some kidney cancers but also by epithelial cells of the bile duct. Patients treated with CAIX specific T-cell developed liver function abnormalities and cholangitis, but unfortunately no cancer regression occurred. 12 patients are part of an ongoing clinical trial that is evaluating the optimal dosage and safety of CAIX specific CAR T-cells (Lamers et al., 2006).

The receptor tyrosine protein kinase ERBB2 specific third-generation CAR T-cells so far also failed to provide a story with a happy ending. Treated patients developed lethal inflammatory cytokine release syndrome in the lungs (Morgan et al., 2010).

More encouraging results so far have been observed in patients treated with disialoganglioside (GD2) specific CAR T-cells. The molecule GD2 which is expressed in neuroblastomas is being tested in pediatric patients and so far, no on target/off tumor toxicity has been detected. In fact, 3/11 patients experienced some tumor regression and two out of these three even sustained complete remission. Patients did not experience any neural toxicity besides pain at the site of tumor. However, as only first-generation CAR T-cells were used for this study, the use of the second-generation CAR technology may enhance the anti-tumor effect as well as toxicity (Louis et al., 2011).

It is evident from existing results that many issues are yet to be resolved. The clinical and toxicity outcomes of CAR T-cell therapy are influenced by tumor type and tumor targeted antigens, by the presence of co-stimulatory molecules, the way CAR T-cells are engineered and also the presence or absence and type of conditional therapy. Engineering CAR T-cells with safety and tissue-specific mechanisms may also be a solution for diminishing off tumor toxic side effects (Guo et al., 2016; Klebanoff et al., 2016).

2.6. Regulating CAR T-cell safety and specificity

To this day the biggest challenge in cancer therapy is to eliminate tumor cells without damaging healthy tissue. To minimize the on target/off tumor CAR T-cell toxicity, many teams have been working on engineering safety and tissue selective mechanisms that would prevent unwanted auto-reactivity of T-cells (Klebanoff et al., 2016). Experimentally tested mechanisms that could enhance the safety and specificity of CAR expressing T-cells are summarized below.

A simple form of CAR T-cell regulation may be achieved by transfecting cells by RNA electroporation. In this way, the receptor is introduced into the cell only temporarily because RNA does not integrate into the host genome. Therefor the expression of CARs on these cells is only short-lived and toxicity, if present, fades spontaneously in a couple of days (Figure 5d) (Zhao et al., 2010).

CAR T-cells may be engineered to co-express a "suicide" gene and become sensitive to the treatment with a prodrug. Thus, in case of CAR-mediated toxicity, it enables the regulation of CAR T-cell activity via induction of apoptosis by application of the prodrug. Good example of such suicide gene is a widely used herpes simplex virus-thymidine kinase. The insertion of this gene into CAR T-cells renders them susceptible to ganciclovir[8] (Figure 5a) (Bonini et al., 1997). An alternative approach is the co-expression of an inducible caspase-9 (iCaps9) construct in CAR T-cells. The human caspase-9 is engineered to be activated after ligand-induced dimerization of CAR construct. This enables specific depletion of activated CAR T-cells that co-express the iCasp9 and undergo induced apoptosis (Figure 4b) (Zhou et al., 2014). Another suicide gene available is the truncated epidermal growth factor receptor (tEGFR). Cells co-expressing tEGFR may undergo antibody-dependent cellular cytotoxicity after the administration of the tEGFR specific antibody cetuximab (Figure 5c) (Wang et al., 2011).

The possibility to eliminate CAR T-cell through induced apoptosis is convenient but the ability to regulate the duration, location and timing of CAR T-cell activity would be even more useful and would enhance the safety of genetically engineered T-cells. Several spatiotemporal control strategies already exist and have been tested. One strategy proposes to co-transduce T-cells with two separate CARs one of which provides a dominant inhibitory signal - iCARs. The dominant inhibitory receptor recognizes an antigen expressed by healthy tissues and its signaling domain is derived from inhibitory molecules such as PD-1 or CTLA-4 (Fedorov et al., 2013; Klebanoff et al., 2016). The other CAR recognizes the tumor antigen, which should not be express on healthy tissues (Figure 5e) (Fedorov et al., 2013). Alternative, but similar approach is the co-transduction of two separate CARs neither of which is sufficient for triggering T-cell activation. These so called "logic-gated" CARs both need to bind antigen in order to activate the cell (Figure 5f) (Klebanoff et al., 2016).

Recently Chia-Yung Wu and collaborators. have developed a more sophisticated mechanism of controlling CAR duration and timing by re-spliting the antigen recognition domain

[8] Antiviral medication

and the signaling domain into two distinct modules. In these "on-switch" CARs, the two modules dimerize only in both the presence of cognate antigen and the dimerizing molecule. Dimerization of the antigen recognition domain and the signaling domain induces signaling and CAR T-cell activation. Activation may be also disrupted by competitive binding of the small molecule dimerizer and again re-induces. This opens up a potential door for pharmacologic regulation of this drug by physicians (Figure 5g) (Klebanoff et al., 2016; Wu et al., 2015). Lastly "masked CARs" have been developed. These receptors have their antigen binding domain blocked by a peptide mask that is attached to a protease substrate. When cells bearing these receptors get into a protease rich environment such as the tumor microenvironment, the protease substrate is cleaved and the mask is released. The antigen binding domain of the receptor is revealed and become operational (Figure 3h) (Klebanoff et al., 2016).

Recently, an outstanding system using a synthetic notch receptor to engineer customized cell response has been introduced by Lim and his group (Morsut et al., 2016). This system has been used by Roybal and colleagues. for engineering „ AND-gate" T-cells that express two distinct receptors which can be activated only after dual antigen recognition. The synthetic notch receptor is expressed constitutively and its activaton drives the expression of the CAR receptor. This „AND-gate" T-cell needs both antigens 1 and 2 to get activated, thus sparing single antigen expressing cells from killing (Morsut et al., 2016; Roybal et al., 2016).

In aggregate, research surrounding the academic and clinical interest in CAR technology is currently booming. Inhere described latest innovations in this field attest to a fast-pacing advancement in constructional design of CAR, CAR transfection approaches, clinical applicability of CAR technology to various types of tumors under well-controlled conditions and with increased biosafety precausions. Given all these features and due to its sensitivity and specificity to recognize relevant molecular structures, in the near future, CAR technology will find even wider applicability not only in the immunotherapy but also in many other areas of translational medicine.

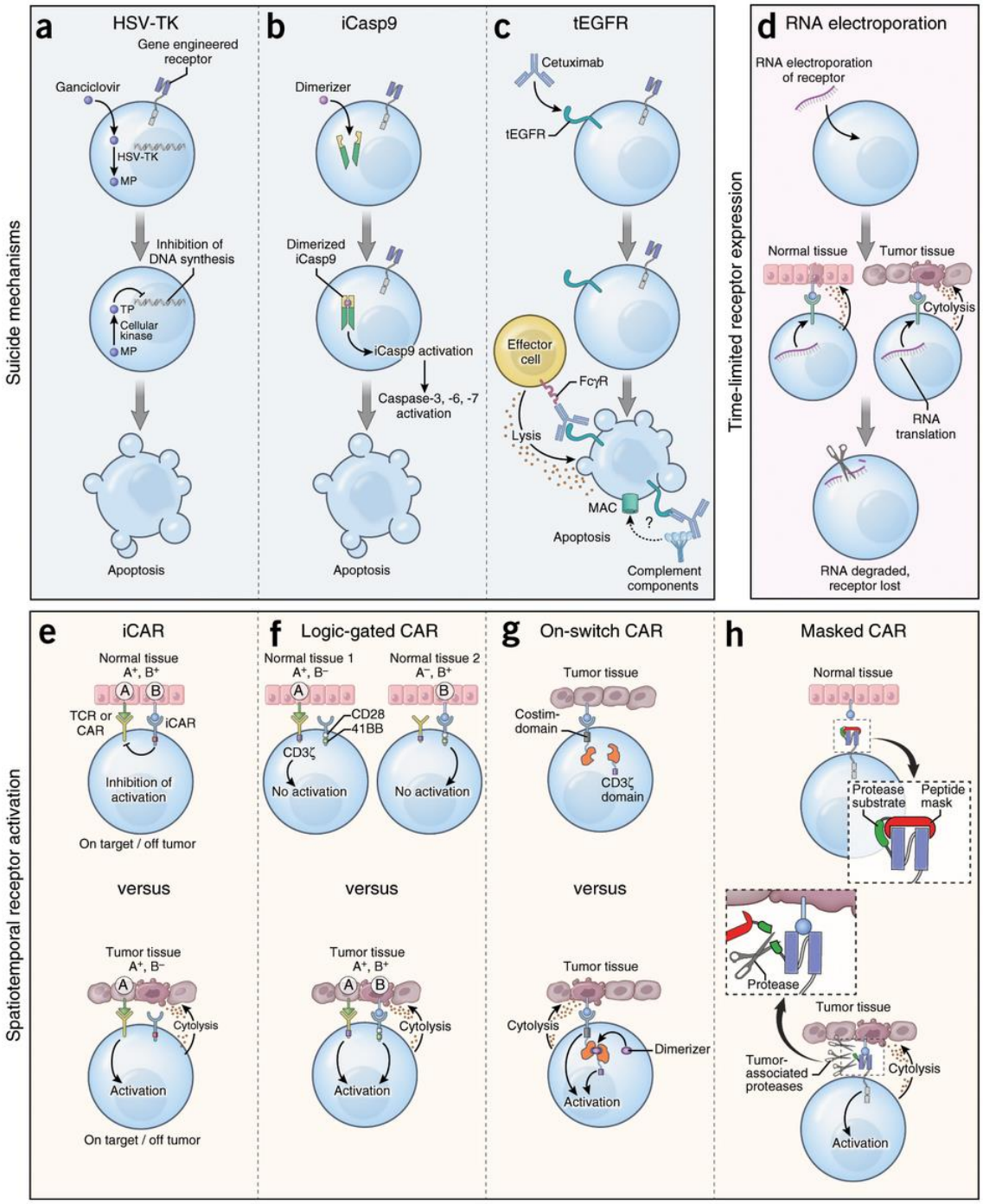

Figure 5: Safety and tissue-selectivity mechanisms that may be inserted into gene-engineered T-cells.

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3. Materials and Methods

3.1. Experiment layout

The overarching goal of the work initiated by MUDr. Pavel Otáhal has been to implement CAR T-cell therapy for B-cell malignancy treatment into the clinics in Czech Republic. Integral part of this goal was to adapt the basic skills and knowledge concerning CAR T-cell engineering. As a part of this effort and the main objective of my thesis was to generate and test the functionality of CD19- and CD20-specific CAR T-cells. An ultimate goal of my work was to prepare a "superCAR" that would carry a construct in which CD19 and CD20 specific CAR sequences are fused into one open reading frame and thus expressed on protein level in an equal stoichiometric ratio. The activation capacity and cytotoxicity of individual CARs and the superCAR T-cells would be then compared.

It is of note that the construction of the superCAR is achieved by a separation of the two individual CAR constructs (CD19 and CD20) by a short viral T2A sequence which ensures that the product of translation is split into two individual peptides accordingly. SuperCAR T-cells would therefore be double specific for both target molecules on malignant B cells - CD19 and CD20. Theoretically, this would endow superCAR T-cells with enhanced potential to recognize MRD and enable the elimination of malignant cells that have lost one of the antigens due to cancer immunoediting. This could minimize the potential of antigen escape by malignant cells.

It is necessary to mention that before I have initiated my work on this project, Dr. Otahal's team was able to design and fully synthesize two CD19-CD20 superCAR constructs: CD20-41BB-FceR1γ-CD19-CD28-CD3 ζ and CD20-CD28-FceR1γ-CD19-41BB-CD3ζ. However, upon their testing, both constructs failed to be expressed in a permissive cell line. Thus, the initial aim of my work was to determine the faulty part of these constructs, find their functional replacement, test CD19 and CD20 constructs individually and only then to attempt to fused these two functional sequences into one working superCAR construct.

3.2. Material

3.2.1. Constructs

Individual constructs specific for the B-lymphocyte antigen CD19 were either cloned out of non-functional superCAR constructs or directly provided as a synthesized sequences by Dr. Otáhal (Table 4). The schematic representation of individual sequences is represented in Figure 6.

contruct	construct origin
CD19-CD28-CD3ζ	cloned out of superCAR CD20-4-1BB-FceR1γ-CD19-28-CD3ζ
CD20-CD28-FceR1γ	cloned out of superCAR CD20-CD28-FceR1γ-CD19-4-1BB-CD3ζ
CD19-4-1BB-CD3ζ	provided by Dr.Pavel Otáhal
CD20-4-1BB-CD3ζ	provided by Dr.Pavel Otáhal

Table 4: Summary of CAR constructs used in the following experiments

All original constructs were provided by Dr. Pavel Otáhal and were then cloned and incorporated in the GFP$^+$ lentiviral vector pWPXLd (Figure 8) instead of GFP.

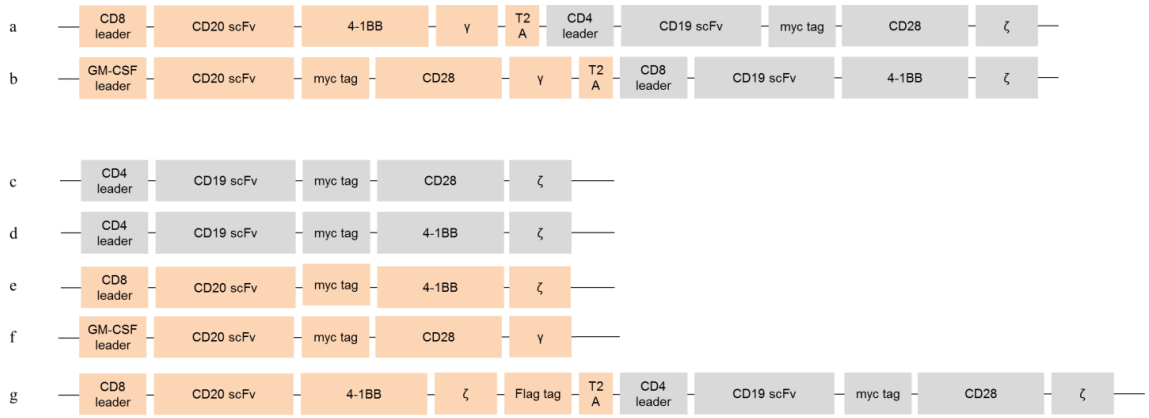


Figure 6: Schematic representation of the sequence modules of individual planned and used constructs.

The first box represents the leader sequence derived from CD4, CD8 or GM-CSF. The following box represents the antigen recognition domain derived from the single chain fragment variable of the anti-CD20 (orange) or anti-CD19 (gray) antibody.4-1BB and CD28 are activation sequences from co-stimulation receptors. Each construct has either ζ or γ activation sequence from the CD3 activation complex in TCR or FceR1 respectively. Myc-tag and flag-tag are tagging sequences enabling antibody detection of individual constructs. a) and b) are original non-functional superCAR constructs. c) and f) have been cloned from a) and b) respectively. d) and e) have been directly provided by Dr. Pavel Otáhal. g) represents the planned new superCAR construct, the structure of which has been designed only after several rounds of testing

Template	Product	F/R primer sequence (5´-> 3´)	Primer name
CD20-CD28-FceR1γ-CD19-4-1BB-ζ	CD20-CD28-FceR1γ	TCATTGGATCCACCGCCATGGTTCTCCTGGTG	CAR-F
		TCATTGAATTCTTACTGTGGAGGCTTCTCGTGCTTCAGGG	CAR20Fc-R
CD20-4-1BB-FceR1γ-CD19-CD28-ζ	CD19-CD28-ζ	TCATTGGATCCATGGGCCGCGGCGTGCCCTTCCGC	CD19-28-z-F
		TCATTGAATTCTTAGCGAGGGGGCAGGGC	CD19-28-z-R
CD20-4-1BB-ζ	CD20-4-1BB-ζ-FlagTag-T2A	CATTAGATCTGAATTCGCCAGCATGGCCCTGCCTGTG	PO20-F
		GAATTCGCCAGCATGGCCCTGCCTG	PO20-F1
		GAAGATCTTCGAATTCGCCAGCATGGCCCTGCCTG	PO20-F2
		TTAGATCTAGGTCCGGGGTTCTCTTCCACGTCGCCGCAGGTCAGCAGGCTGCCCCGGCCCTCGCCGGAGCCGCTGCGCTTGCGCCGCTTGTCGTCATCGTCTTTGTAGTCTCTGGGGGGCAGGGCCTGCATGTGCAGGG	PO20-R
		TCT GGG GGG CAG GGC CTG CAT GTG CAG	PO20-Rev1Seq

Table 5: Sets of primers used for amplification and cloning of CAR constructs

All constructs were sub-cloned into the lentiviral vector pWPXLd. Forward (F) and reverse (R) primers are in red and green colors, respectively.

Annealing region	F/R primer sequence (5´-> 3´)	Primer name
5´and 3´end of MCS in the pJET cloning vector	GAGCAGGTTCCATTCATTGTT	TUPOF
	GTTAGCATAGTTCTTAATATAAGTT	TUPOR
Inside CAR20-4-1BB-ζ	AAGTGGCGGAGGGGGATCCGATAT	PO20_SP-F1
	CCTAGACCTCCAACACCCGCCCCT	PO20_SP-F2
	TGCAGGACATCTTCACGGAG	PO20_SP-R1
3´end of CAR20-4-1BB-ζ	TCT GGG GGG CAG GGC CTG CAT GTG CAG	PO20-Rev1-Seq
	GGA GCA ACA TAG TTA AGA ATA CC	PO20-Rev2-Seq
inside pWPXLd	CAT TCT CAA GCC TCA GAC AGT GG	pWPXLd F1
	GGA AAG AAT AGT AGA CAT AAT AGC	pWPXLd F2

Table 6: Sequencing primers

Sequencing primers used in different combinations for the verification of amplified sequences during cloning procedures of individual CAR constructs into the cloning vector pJET (Figure 7) and the expression lentiviral vector pWPXLd (Figure 8).

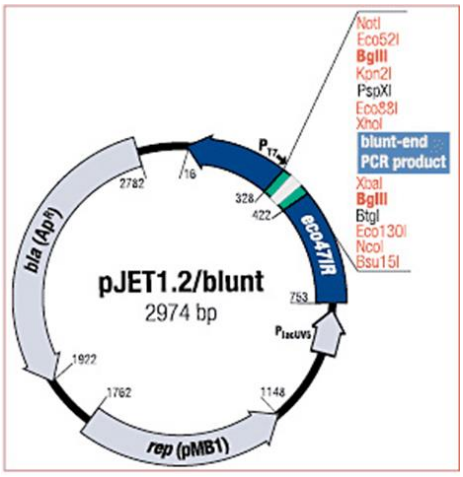


Figure 7: pJET1.2 blunt vector map

The individual CAR blunt-end PCR products were cloned into the pJET vector which carries the selection gene for ampicillin resistance.

Source: https://www.thermofisher.com/order/catalog/product/K1231)

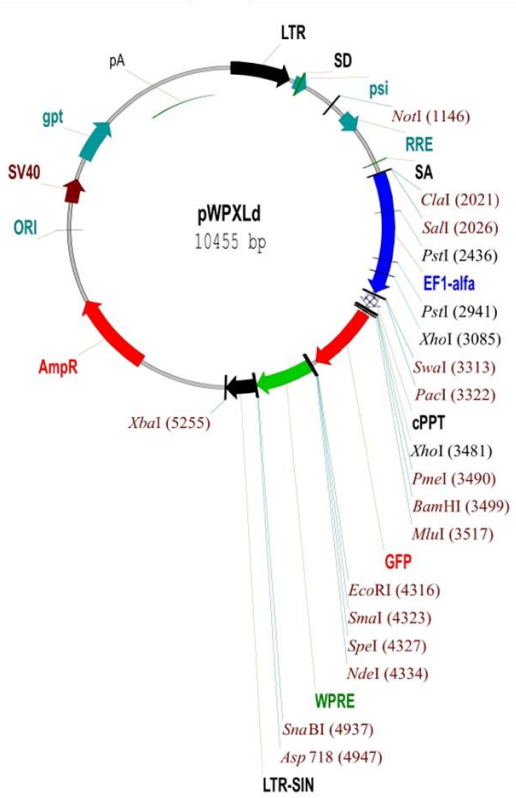


Figure 8: pWPXLd lentiviral vector map

The individual CAR constructs were cloned to replace GFP sequence. The expression vector carries the ampicillin resistance gene.

3.2.2. Cell lines and culture conditions

All cell lines were maintained in an appropriate medium (Table 7) supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin and cultivated at 37°C with 5% CO_2, if not stated otherwise.

Cell line	Cell type	Cultivation medium
HEK293T	human embryonich kidney cells	DMEM
Jurkat B10	immortalized human T-lyphocytes	RPMI 1640
RAJI	human B-cell lymphoma	RPMI 1640
$BW5147^{NFAT\text{-}GFP}$	mouse thymus T-cell lymphoma	IMDM
$Jurkat^{CAR}$	immortalized human T-lyphocytes	RPMI 1640
$BW5147^{NFAT\text{-}GFP\text{-}CAR}$	mouse thymus T-cell lymphoma	IMDM

Table 7: Cell lines and their culture mediums used in described experiments.

3.2.3. Antibodies

In below described experiments several methods requiring the use of monoclonal antibodies were used. Used antibodies and their specificities are summarized in Table 8.

Antigen	Clone	Host	Reactivity	Conjugate	Manufacturer	Method
primary antibodies						
CD69	FN50	mouse	human	AF647	Exbio	FACS
CD20	LT20	mouse	human	APC	Exbio	FACS
CD3	MEM57	mouse	human	PE	Exbio	FACS
CD28			human	purified	kind gift from prof.Hořejší	activation
CD19	LT19	mouse	human	FITC	Exbio	FACS
GAPDH		rabbit	human/mouse /rat	purified	Sigma-Aldrich	WB
Myc-Tag		mouse		purified	Cell Signaling Technology	WB
secondary antibodies						
F(ab´)$_2$		goat	mouse	AF647	Jackson ImmunoResearch	FACS
IgG light chain		goat	mouse	horsradish-peroxidase	Jackson ImmunoResearch	WB
rabbit mAb		goat	rabbit	horsradish-peroxidase	Biorad	WB

Table 8: Primary and secondary monoclonal antibodies used in described experiments.

3.3. Methods

3.3.1. Polymerase chain reaction

3.3.1.1. Cloning of individual CARs from non-functional superCAR constructs

The nonfunctional superCAR constructs were used as templates for polymerase chain reaction (PCR) to obtain individual CAR sequences (Table 4). Primers specific for individual constructs (Table 5) were used for their PCR amplification reaction (Table 9).

CAR CD20-CD28-FceR1γ was cloned out of CAR CD20-CD28-FceR1γ-CD19-4-1BB-ζ using primers CAR-F and CAR20Fc-R. CD19-CD28-ζ was cloned out of CAR CD20-4-1BB-FceR1γ-CD19-28-CD3ζ using primers CD19-28-z-F and CD19-28-z-R (Table 5).

Components	25 µL reaction
5x Q5 reaction buffer	5 µL
10mM dNTPs	0.5 µL
10µM forward primer	1.25 µL
10µM reverse primer	1.25 µL
Template DNA (10 ng)	1 µL
5x high-fidelity DNA polymerase	0.25µL
5x Q5 high enhancer	5µL
Nuclease free water	10.75µL

Steps	Temperature	Time
Initial denaturation	98°C	30 sec
35 cycles		
Denaturation	98°C	7 sec
Annealing	62°C	20 sec
Elongation	72°C	50 sec
Final extention	72°C	2 min

Table 9: PCR characteristics.

The properties of PCR used for the cloning of CD20-CD28-FceR1γ and CD19-CD28-ζ out of their appropriate superCAR. Q5 high-fidelity DNA polymerase was used for amplification (New England BioLabs Inc.,Ipswich, Massachusetts.

3.3.1.2. Generation of a new superCAR

For financial reasons, we had to generate a new superCAR construct by using general methods of molecular cloning, rather than by direct synthesis. The simplified cloning strategy is described in Figure 9. The CAR CD20-4-1BB-ζ construct in pWPXLd was cloned out of the lentiviral expression vector (Figure 8) using a two-step PCR protocol (Table 10). Primers PO20-F and PO20-Rev1Seq were used for the first PCR reaction step (Table 5; Table 10). The PCR product of correct size was resolved by agarose gel electrophoresis, excised from the gel and isolated by Agarose Gel Extraction Kit (Jena Bioscience GmbH). The isolated

product was used as a template for the second PCR amplification step with primers PO20-F and PO20-R (Table 5; Table 10).

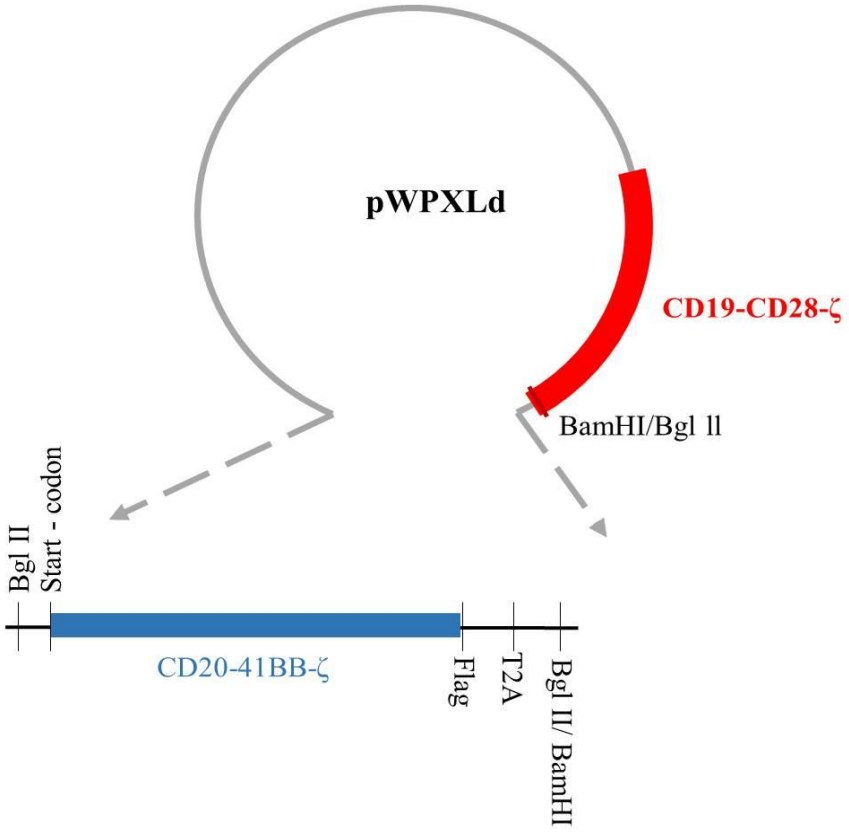


Figure 9: Simplified scheme of the cloning strategy for the generation of a new superCAR construct.

CD20-4-1BB-ζ is cloned out of the pWPXLd expression vector in a two-step PCR. In the first PCR step, the CD20-4-1BB-ζ fragment is cloned out of the expression vector using primers PO20-F and PO20-Rev1Seq. In the second PCR step the product from the first PCR is used as a template. Primers PO20-F and PO20-R were used. The forward primer adds to the pre-existing CD-20-4-1BB-ζ sequence the Bgl II restriction site at the 5´end. The reverse primer adds to the 3´end a flag-tag to distinguish CD20-4-1BB-ζ from the myc-taged CD19-28-ζ. The reverse primer also introduces the T2A sequence which enables the transcription of the superCAR construct in one open reading frame but the translation and expression of the individual CARs as separate polypeptides. At the far 3´end, the reverse primer also generates a Bgl II restriction site. This final fragment is ligated in front of the pre-existing CD19-CD28-ζ in pWPXLd using the Bgl II and BamHI restriction sites which are fully complementary.

Components	25µL reaction
5x Q5 reaction buffer	5µL
10mM dNTPs	0.5µL
10µM forward primer	1.25µL
10µM reverse primer	1.25µL
Template DNA (10ng)	1µL
5x High fidelity DNA polymerase	0.25µL
5x Q5 enhancer	5µL
Nuclease free water	10.75µL

Steps	Temperature	Time
Initial denaturation	98°C	60 sec
35 cycles		
Denaturation	98°C	7 sec
Annealing	60°C	20 sec
Elongation	72°C	90 sec
Final extenstion	72°C	120 sec

Table 10: PCR characteristics.

The PCR properties used for the generation of CD20-4-1BB-ζ-CD19-CD28-ζ superCAR.

3.3.2. Ligation into the cloning vector

The size of blunt-ended PCR products were verified by agarose gel electrophoresis and ligated into the pJET cloning vector (Thermo Scientific, Waltham, Massachusetts) at room temperature (RT) for 5 minutes (Table 11). Then, 4 µl of ligation mixture were used for heat shock-mediated transformation of bacteria which were then spread on Luria-Bertani (LB) agar plates with ampicilin (Amp) and cultivated upside down overnight at 37°C. Single colonies were selected, resuspended in LB medium with Amp and cultivated overnight by shaking at 37°C. Plasmid isolation was performed by Zyppy™ Plasmid Miniprep Kit. DNA was eluted into 15 µL of RNas-free water and concentration was determined by nanodrop ND-1000 spectrophotometr.

Components	Volume
2x reaction buffer	5 µL
PCR product	1 µL
pJET1.2/ blunt cloning vector (50 ng/µL)	0.5 µL
Nuclease free water	3 µL
T4 DNA ligase	0.5 µL

Table 11: Composition of ligation reaction.

Individual CAR PCR products were ligated into the pJET cloning vector. Reaction was set at room temperature (RT) for 5 minutes. (Thermo Scientific, Waltham, Massachusetts)

3.3.3. Restriction enzyme digestion protocol

Correct ligation of individual CAR constructs into the pJET cloning vector was verified by enzyme digestion (Table 12 and 13). Restriction enzymes and buffers were provided by Thermo Scientific, Waltham, Massachusetts.

Components	Volume
DNA	2µL (300ng DNA)
enzyme EcoRI	1µL
enzyme BamHI	1µL
buffer Tango	2µL
water	4µL

Table 12: Reaction properties for the restriction of CD19-CD28-ζ and CD20-CD28-FceR1γ.

Control restriction reaction of CD19-CD28-ζ and CD20-CD28-FceR1γ out of the pJET cloning vector was performed by restriction enzymes BamHI and EcoRI at 37°C for 1 hour.

Components	Volume
DNA	0,5µL (300ng DNA)
enzyme BglII	1µL
buffer O	1µL
water	7.5µL

Table 13: Reaction properties for the restriction of CD20-4-1BB-ζ.

Control restriction reaction of CD20-4-1BB-ζ out of the pJET cloning vector was performed by the restriction enzymes Bgl-II at 37°C for 1 hour.

Restriction was verified by agarose gel electrophoresis and products corresponding to the expected molecular size of individual CAR constructs (CD19-CD28-ζ = 1542 bp; CD20-CD28-FceR1γ = 1281bp; CD20-4-1BB-ζ = 1522bp) were sent for sequencing. Sequences were analyzed using the program BioEdit. Products correctly ligated into the pJET cloning vector were again cut by restriction enzymes BamHI and EcoRI (CD19-CD28-ζ and CD20-4-1BB-FceR1γ) and Bgl-II (CD20-4-1BB-ζ). Adequate fragments were separated from the pJET vector

by agarose gel electrophoresis. DNA was then isolated by Agarose Gel Extraction Kit (Jena Bioscience GmbH) and its concentration determined by nanodrop. CAR fragment ligation into the lentiviral expression vector pWPXLd was done analogously to the cloning into the pJET cloning vector using 1:3 molar ratio as mentioned above.

3.3.4. CAR transfection by lipofection

To determine correct CAR cell surface expression, the HEK293T cell line was transfected by lipofection using the protocol provided by SigmaGen Laboratories for their LipoJet™ In Vitro DNA and siRNA Transfection Kit.

3.3.5. Western-blot

To verify CAR expression on protein level, HEK293T were transfected with individual CAR constructs and their cell lysates analyzed by western blot. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamid resolving gel and 4% acrylamic stacking gel) and blotted to a polyvinylidene fluoride (PVDF) membrane. CAR constructs containing a myc-taged intracellular signaling domain were probed with the primary mouse myc-tag specific antibody and visualized by goat anti-mouse IgG light chain specific secondary antibody conjugated with horseradish-peroxidase (Table 8). Enhanced chemiluminescent substrate (ECL) 1 and ECL2 were used as horseradish-peroxidase substrate (Thermo Scientific, Waltham, Massachusetts).

3.3.6. Lentiviral vector preparation and engineering of CAR T-cells

For the generation of a T-cell line stably expressing individual CAR constructs, Jurkat cells were genetically modified using a second-generation self-inactivation lentiviral vector based system. Infectious viral particles were generated in HEK293T cells. HEK293T cells were plated on day 0 in order to obtain approximately 80% confluency on day 1. Cells were co-transfected on day 1 with the transfection mixture (Table 14) containing transgene expression vector (CD19-CD28-ζ; CD19-4-1BB-ζ; CD20-4-1-BB-ζ; empty vector pWPXLd), and the viral packaging plasmids (VSV-G; GagPol) (provided with generosity by Meritxell Alberich Jorda, Ph.D.) using the transfection reagent polyethylenimin (PEI). Cells were incubated at 37°C supplemented with 3% CO_2. On day 2, i.e. 24 hours after transfection, the medium was carefully replenished. Viral particles containing infection medium was collected on day 2 and 3 after transfection, centrifuged at 3000 rpm for 5min and used to infect Jurkat cells at 37°C supplemented with 5% CO_2 for 3h. After 3h, infectious medium was replaced with regular medium (Table 7).

Components	Volume
DMEM (no ATB, no FBS)	100 µL
Gag/pol	1 µg
VSV-G	1 µg
construct	1 µg
PEI	9 µL

Table 14: Components and volumes of the transfection mixture.

The transfection mixture was prepared and left at RT for 20min and then applied drop-wise onto cells. The volumes correspond to 450 000 HEK293T cells plated on day 0 on a 6-well plate resulting in 80% confluency on day 1.

3.3.7. CAR transfection by electroporation

The mouse T-cell line BW5147 in which NFAT is fused with the green fluorescent protein (GFP) was transfected with individual CAR constructs by electroporation. 15-20 million cells were electroporated in 300µL DMEM medium supplemented with 10% FBS and in the absence of antibiotics. DNA concentration was 1.5 µg per million cells and voltage was 270V. Electroporation was performed using the BTX-ECM830 electroporator (BioTech, Prague, Czech Republic).

3.3.8. Fluorescence-activated cell sorting

The expression of individual CAR constructs and NFAT-CAR T-cell activation was confirmed by fluorescence-activated cell sorting (FACS). Cell suspension was stained in an appropriate amount of fluorophore-conjugated antibodies (Table 8) for 25 minutes at 4ºC in the dark. Cells were washed twice with PBS supplemented with 3% phosphate-buffered saline (FBS) to remove unbound antibodies that would cause a false positive signal. Cells were also stained by Hoechst 33258 (eBioscience, San Diego, California) to label dead cells for their elimination from further analysis. All FACS data were derived from gating on live single cells. Cells were analyzed using the LSR II flow cytometer (BD, Biosciences, San José, California) and obtained data was processed using FlowJo 9.9 software (Tree Star, Ashland Oregon). To obtain a pure population of CAR expressing T-cells, cells were sorted by BD Influx Cell Sorter using the above described staining protocol (BD Biosciences, San José, California).

4. Results

4.1. General consideration

CAR T-cells are being tested in phase I/II clinical trials all over the world mainly in the United States of America, China and some European countries (Appendix). Czech Republic however is not one of them. The overarching goal of this work was, in close collaboration with Dr. Pavel Otáhal, to complete a preparatory phase for implementing CAR T-cell therapy for hematological malignancies into the Czech clinic, notably, to prepare a functional CAR constructs targeting CD19 and CD20 markers on malignant B cells.

Specific aims were to verify the functionality of individual CAR constructs derived from the original non-functional superCAR constructs and attempt to create a new functional superCAR that would endow T-cells with dual specificity for both CD19 and CD20 antigens. Below, these specific aims are arranged as chronological list of laboratory experiments:

a. Generation of the following CAR constructs:

   CD19-CD28-ζ

   CD19-4-1BB- ζ (provided by Dr. Pavel Otáhal)

   CD20-CD28-FceR1γ

   CD20-4-1BB-ζ (provided by Dr. Pavel Otáhal)

   CD20-4-1BB-ζ-CD19-CD28-ζ

b. Generation of Jurkat T cell lines expressing individual CAR constructs (Jurkat$^{CAR)}$.

c. Verification of the functionality of individual CAR construct by several functional assays.

d. Expression of individual CARs in patients T-cells and comparison of their effector functions.

4.2. Generation of CAR constructs

4.2.1. Cloning the independent CAR constructs

Generally, the constructs were prepared by amplifying relevant sequences from originally prepared but non-functional superCAR constructs as shown in Figure 10 and 11.

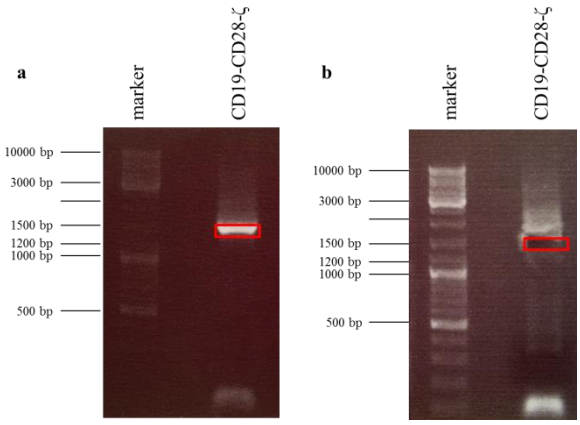


Figure 10: CD19-CD28-ζ PCR product.

a) Red rectangle denotes CD19-CD28-ζ PCR product (1542bp) amplified from CD20-4-1BB-FceR1γ-CD19-CD28-ζ superCAR template with primers CD19-CD28-z-F and CD19-CD28-z-R (Table 5). b) The product corresponding to the molecular size of 1542bp was cut out of the agarose gel (empty red rectangle), DNA was isolated and ligated into the cloning vector pJET.

Analogously to CD19-CD28-ζ cloning (Figure 10) the CD20-CD28-FceR1γ PCR product was amplified from the non-functional supercar (Figure 11).

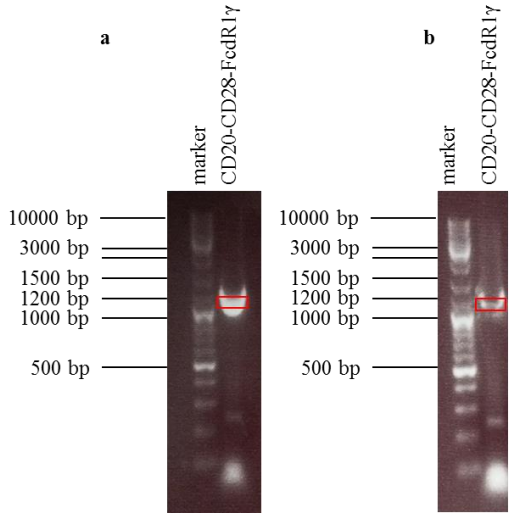


Figure 11: CD20-CD28-FceR1γ PCR product.

a) Red rectangle denotes CD20-CD28-FceR1γ PCR product (1281bp) amplified from CD20-CD28-FceR1γ-CD19-4-1BB-ζ superCAR with primers CAR-F and CARFc-R (Table 5). b) The product corresponding to the molecular size of 1281bp was cut out of the agarose gel (empty red rectangle), DNA was isolated and ligated into the cloning vector pJET.

4.2.2. SuperCAR cloning

In order to construct a new superCAR, the faulty part of CAR sequence had to be identified. Then, the intention was to replace these parts with functional CAR sequences that were chosen after testing for making a fused CD20-CD19 superCAR construct. Experiments highlighted below show that suitable candidates for superCAR construction were CD19-CD28-ζ and CD20-4-1BB-ζ. Therefore, we had decided to amplify one of these sequences, notably CD20-4-1BB-ζ, and then fused it by ligation with CD19-CD28-ζ template (Figure 9 and Figure 12). Several additional sequences needed to be added to the construct to ensure optimal stoichiometry of translation, surface expression and detectability (for cloning strategy details see Material and Methods).

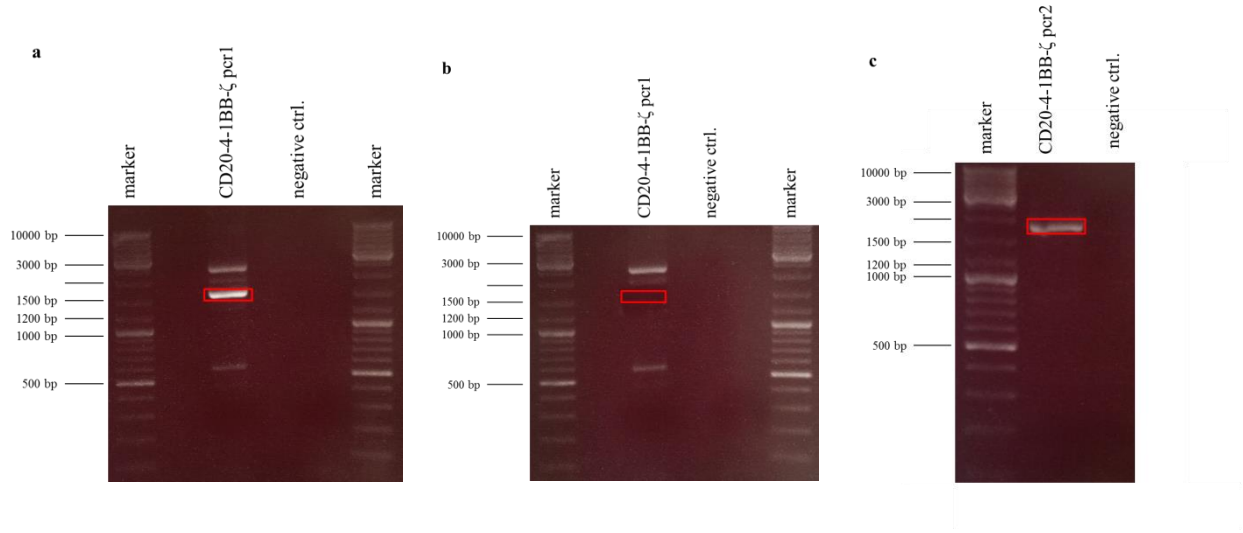


Figure 12: CD20-4-1BB-ζ PCR enhancement.

a) The fragment CD20-4-1BB-ζ was cloned out of the lentiviral vector pWPXLd in the first round of the two step using primers PO20-F and PO20-Rev1Seq (Table 5). b) Because the PCR product is a mixture of specific and non-specific fragments, the fragment corresponding to the size of 1521bp was cut out of the agarose gel, DNA was isolated and used for the second PCR step. c) The second PCR using primers PO20-F and PO20-R generated the fragment CD20-4-1BB-ζ enriched for the sequences needed for the construction of the superCAR with appropriate restriction sites as well as Flag-tag and T2A sequences (Figure 9).

4.3. CAR construct expression on protein level

The individual CAR constructs were expressed in HEK293T (for details see Materials and Methods) and their expression on the protein level was first verified by western blot (for details see Materials and Methods). The result presented below in figure 13 suggests that the superCAR co-expressing CD20-CD28-FcεR1γ can´t be expressed due to faulty expression of this construct. Other CAR constructs incorporating the CD3ζ signaling component were detectable on protein level.

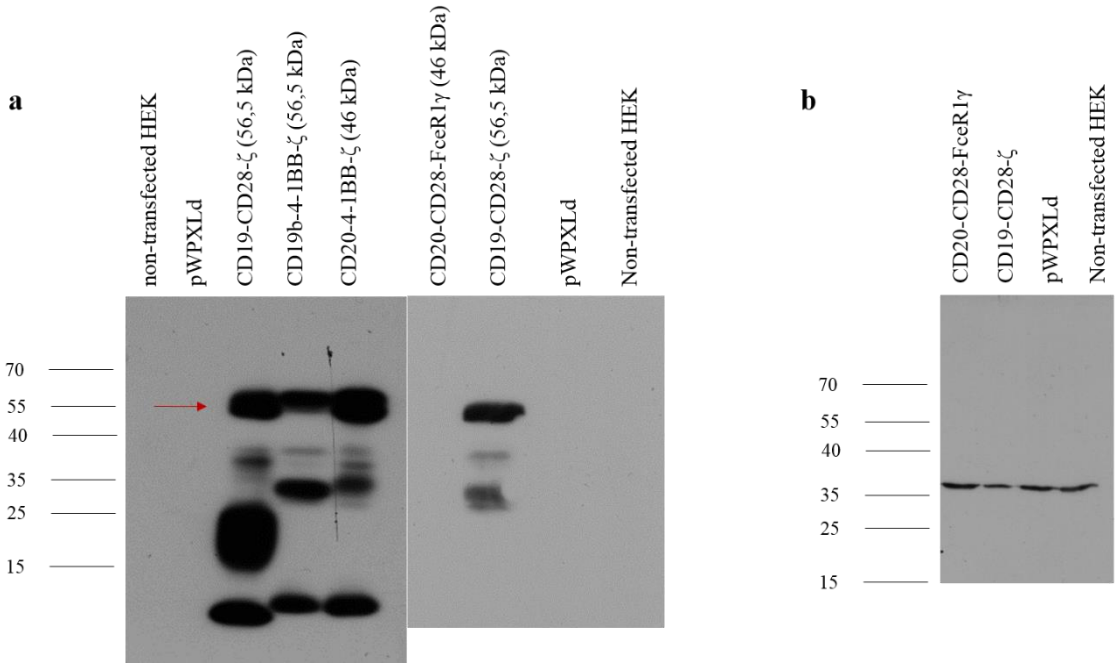


Figure 13: Western-blot showing the expression of individual CAR constructs.

a) CD19-CD28-ζ(56,5kDa), CD19-4-1BB-ζ (56,5kDa), and CD20-4-1BB-ζ(46kDa), all containing a Myc-tag- intracellular domain were readily detected in the HEK293T cell line (bands corresponding to the red arrow). The expression of CD20-CD28-FceR1γ, which also has an incorporated Myc-tag sequence, was not detected on protein level. Non-transfected HEK293T cells and cells transfected with the empty vector pWPXLd were used as negative controls. The non-specific bands are considered as products of degradation. b) The housekeeping gene GAPDH (36kDa) was used as a loading control.

4.4. Lentivirus vector production

Lentiviruses carrying the CD19-CD28-ζ, CD19-4-1BB-ζ, CD20-4-1BB-ζ or GFP in the empty vector pWPXLd were produced in HEK293T cell line (view Material and Methods). Transfection efficiency was verified by FACS analysis of the virus producing cells. The antigen specific domain of the CAR construct is the scFv derived from a CD19 or CD20 specific mouse antibody, therefore the goat-anti-mouse Alexa 647 conjugated mAb was used to detect surface expression of CARs. As illustrated in figure 14, the expression of individual CAR constructs in infected HEK 293T cells was ≥88% and therefore succesfull production of functional

lentiviral particles. Non-transfected cells and cells transfected with empty vector were used as negative controls.

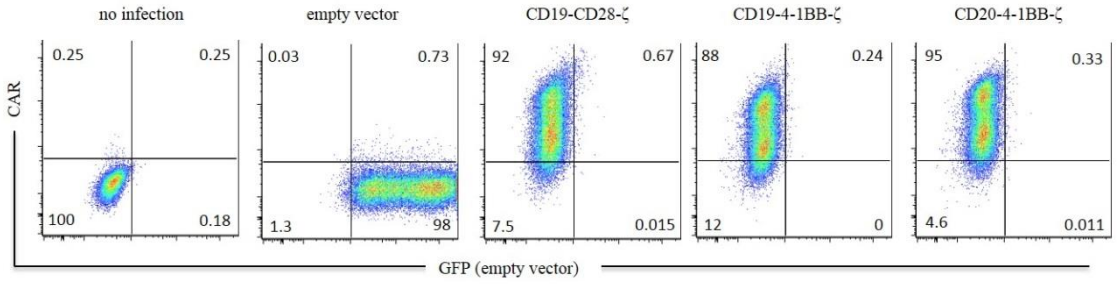


Figure 14: CAR expression in the virus producing HEK293T cell line.

HEK293T cells were stained by goat anti-mouse F(ab')$_2$ specific antibody. The presence of empty lentiviral pWPXLd vector was detected due to GFP expression. Cloning of CAR constructs into these vectors replaces GFP and thus cells become GFP$^-$. Cells that have not been transduced were negative for the CAR construct. 98% of cells transfected with the pWPXLd empty vector were GFP$^+$ but CAR$^-$. 92% of cells transfected with CD19-CD28-ζ, 88% of cells transfected with CD19-4-1BB-ζ, and 95% of cells transfected with CD20-4-1BB-ζ, were CAR$^+$.

4.5. CAR expression in T cells

Jurkat T-cells often serve as a surrogate $CD3^+$ T cell population for testing various aspects of T cell physiology and are often used to test the efficiency of CAR constructs (Posey et al., 2016; Wu et al., 2015). Here, Jurkat T cells were infected with indicated lentivirus CAR constructs (Figure 15). Non-transfected Jurkat T cells and Jurkat cells transfected with an empty vector were used as negative controls. The infection efficacy of Jurkat cells ranged from 10-14%. Transfection efficiency of Jurkat cells by empty vector was 3 times higher than transfection by individual CAR constructs. This is likely caused by a larger size of the constructs carrying CAR transgene (Figure 15).

As shown in Figure 15, 25% of non-transfected cells were $CD3^+$ and none of them expressed the CAR construct. 14% of cell transfected with CD19-CD28-ζ, 10% of cells transfected with CD19-4-1BB-ζ and 9.8% of cell transfected with CD20-4-1BB-ζ, were double positive. Out of the cells transfected with pWPXLd empty vector 31% were double positive for empty vector (GFP^+ cells) and CD3. Overall, the low expression of CD3 by Jurkat cells is likely due to a bad quality of the used aliquot which spontaneously lost CD3 expression. It has also been reported that low expression of CD3 may be associated with the relatively low lentiviral transduction efficiency (Wherry, 2011). In all subsequent functional experiments $CD3^+CAR^+$ Jurkat cells were sorted out, and thus this issue could be neglected.

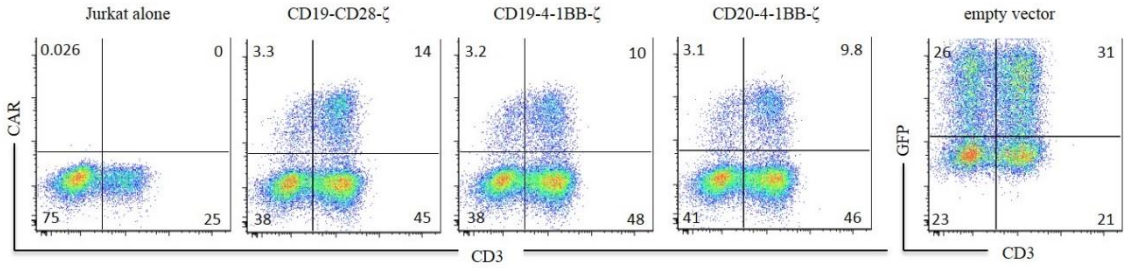


Figure 15: CAR expression in transfected Jurkat cells.

Transfected and non-transfected cells were stained by the goat anti-mouse F(ab)′₂ specific antibody and by PE conjugated CD3 specific antibody. It is of note that only those empty vector transduced Jurkat cells were considered as positive which expressed intermediate- to high levels of GFP.

4.6. CD69 expression

To verify CAR T-cell activation we measured the expression of activation molecule CD69 by Jurkat T-cells after stimulation with Raji B-cells expressing CAR-targeted antigens, CD19 and CD20. CD69 is a transmembrane C-type lectin and is considered as one of the first gene expression responses after T-cell activation (Simms and Ellis, 1996). Jurkat cells expressing individual CAR constructs were co-cultured overnight with Raji ($CD19^{+}CD20^{+}$) target cells (Figure 16) at a radio 1:4. Cells were then pelleted and stained for CD69 and CD3 (Figure 17). Jurkat cells not expressing CARs and Jurkat cell expressing empty vector were used as negative controls. Activated Jurkat cells by anti-CD3 and anti-CD28 mAb was used as a positive control (dot plot not shown).

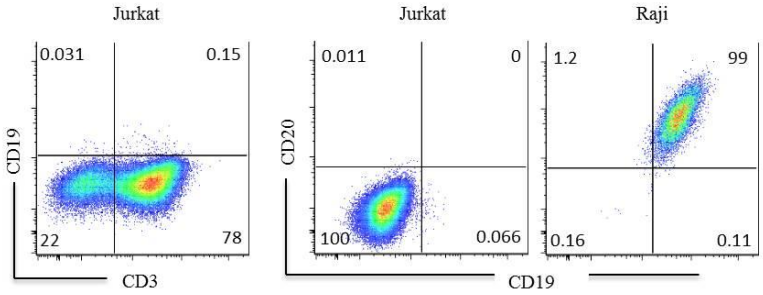


Figure 16: CD19 and CD20 expression by Raji target cells.

99% of Raji target cells are double positive for the target molecules CD19 and CD20. Jurkat cells were used as a negative control.

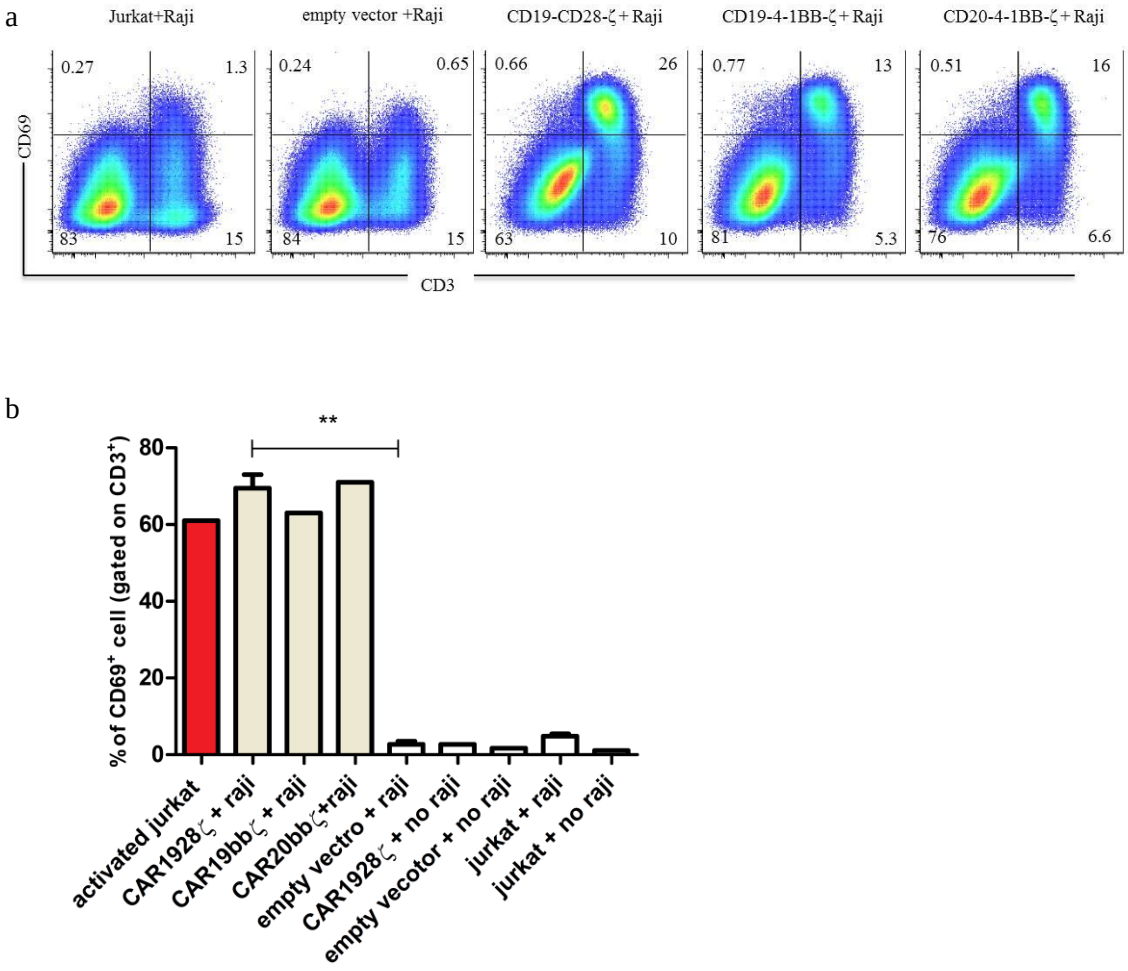
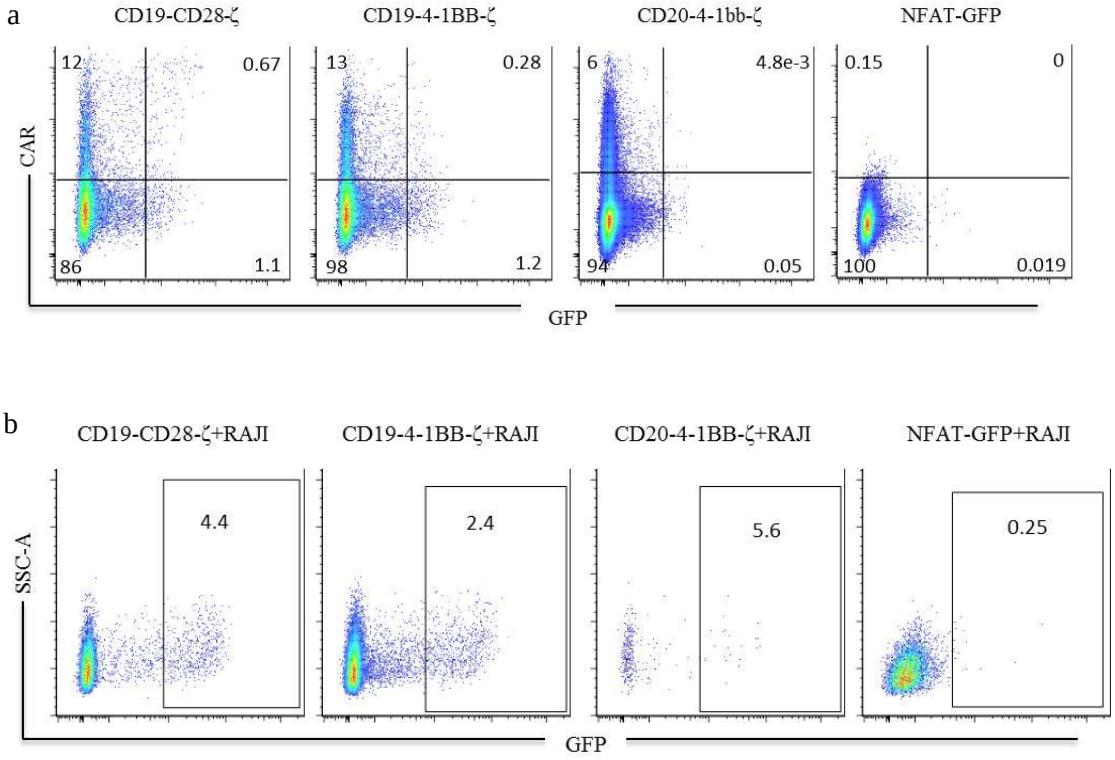


Figure 17: CD69 expression by activated CAR T-cells after co-cultivation with $CD19^{+}CD20^{+}$ Raji target cells.

a) After overnight co-cultivation, cell mixture was stained by anti-CD69 Alexa647 and anti-CD3 PE to distinguish Jurka from Raji cells. There was a noticeable shift from $CD3^{+}CD69^{low}$ to $CD3^{+}CD69^{high}$ population. 26% of all cells were double positive after Jurkat-CD19-CD28-ζ activation by target cells. Co-cultivation of Jurkat-CD19-4-1bb ζ with Raji cells cause activation of 13% of all cells and in the experiment with Jurkat-CD20-4-1bb ζ the percentage of activated cells was 16%. b) The bar-graph quantifying the experiment shown in (a) in which the proportion of activated $CD69^{+}$ cells is related to the population of $CD3^{+}$ Jurkat T cells only; those co-culture experiments which show mean with SD were performed 3 times (n=3), those shown as mean value only, twice (n=2).

4.7. NFAT-GFP activation assay

To verify the functionality of the CAR constructs we performed another activation experiment using the mouse thymoma cell line BW5147 which has the transcription factor NFAT fused with GFP (further referred to as NFAT-GFP cells) (Figure 18b and c). These cells were electroporated by individual CAR constructs (for details see Materials and Methods). CAR expression after electroporation was determined 24h later (Figure 18a). CAR^+ cells were sorted and co-cultivated overnight with target Raji cells (Figure 16). Electroporated NFAT-GFP with the absence of CAR construct were used as a negative control. The empty vector pWPXLd could not be used as a negative control because it carries the GFP reporter gene.



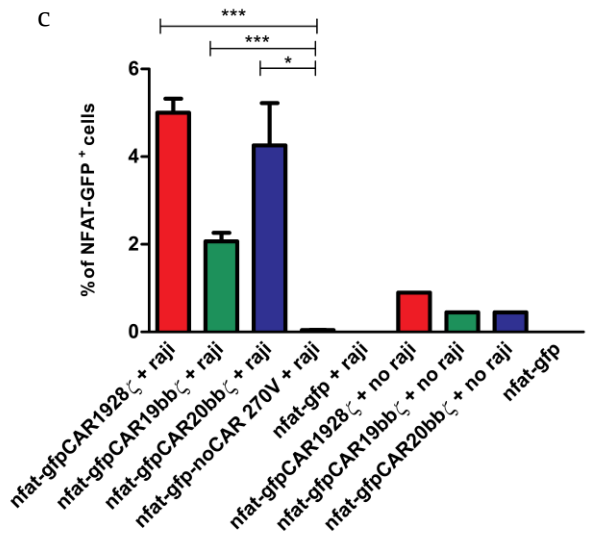


Figure 18: NFAT-GFP electroporation and activation.

a) Electroporated cells were stained with the CAR specific antibody anti-F(ab)´$_2$. 12%, 13% and 6% of cells were positive for CD19-CD28-ζ, CD19-4-1BB-ζ and CD20-4-1BB-ζ construct, respectively. The electroporation efficiency was lower than expected; CAR$^+$ cells were therefore sorted and afterwards co-cultivated with Raji cells at ration 1:4. b) GFP expression after overnight co-cultivation with Raji cells revealing NFAT activation in CAR positive cells. Samples electroporated with individual CAR constructs were gated on CAR$^+$ cells and GFP expression was analyzed. We could detect a distinct population of CAR$^+$GFP$^+$cells, however the percentages were very low - 4.4%, 2.3% and 5.6% for CD19-CD28-ζ, CD1-4-1BB-ζ and CD20-4-1BB-ζ, respectively. Co-cultivation of CAR$^-$ cells with Raji cells served as a negative control. This sample was not gated on CAR$^+$ cells. c) Bar-graph showing the percentage (mean with SD; n=3 for CD19-CD28-ζ, CD19-4-1bb-ζ and n=2 for CD2-4-1BB-ζ) of NFAT-GFP$^+$ cells (gated on CAR$^+$ cells) after activation with target Raji cells.

We also wanted to see whether the expression of two distinct CAR constructs by a single cell would have some sort of effect on T-cell activation. At this point we did not yet have the superCAR construct prepared, so to mimic the potential activity of double specific cells, we tried an alternative approach - electroporation of the NFAT-GFP cell line by two distinct CAR constructs simultaneously (Figure 19).

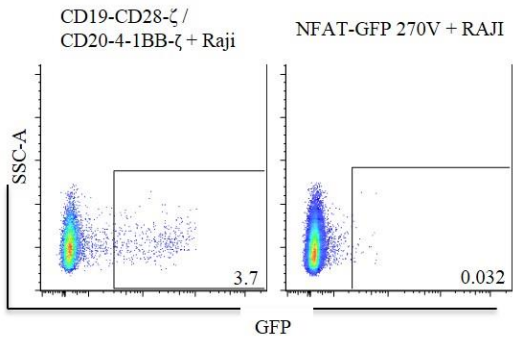


Figure 19: NFAT-GFP double CAR expressing cells.

NFAT-GFP cells were electroporated with 270V by two constructs simultaneously to mimic the effect of the potential superCAR construct. Cells were electroporated with the constructs used for superCAR generation – CD19-CD28-ζ and CD20-4-1BB-ζ. The cultivation of NFAT-GFP cells that received 270V with Raji cells were used as a negative control.

5. Discussion

Adoptive therapy with CD19 specific CARs has met great success in the clinics for the treatment of hematological malignancies (Sadelain, 2015). However it has been reported that treatment with CD19 specific CAR T-cell enhances the appearance of $CD19^-$ tumor cells (Grupp et al., 2013; Maude et al., 2014). The idea of generating a double specific CAR that would be specific for the antigen CD19 and also for the alternative B-cell specific surface antigen CD20, logically came to mind. This mechanism would greatly diminish the possible escape of tumor cells that have lost the antigen CD19. The two superCAR constructs designed by Dr. Otáhal, (Table 4, Figure 6) however failed to be expressed on the cell surface of permissive cells. In order to at least partially reveal the reason why these constructs are not expressed, we cloned the individual CAR constructs and tested their protein expression independently. CD19-CD28-ζ, CD19-4-1BB- ζ, CD20-CD28-FceR1γ, CD20-4-1BB-ζ, were transfected into the Jurkat T-cell line, but among these, only CD20-CD28-FceR1γ failed to be expressed (Figure 13). While we did not analyzed the reason behind such failure, the question, whether the incorporation of an alternative activation domain which would replace FceR1γ segment, has been raised. The use of the γ signaling chain from the FcεR1 in CARs has been previously reported (Eshhar et al., 1993). Given that the protein expression of γ fragment-derived from FcεR1 should not represent a problem, we can't exclude that a technical error was likely to cause this outcome. As an *in vivo* comparative study between the γ-chain and CD3ζ chain has shown that the latter is a much more potent activator of CAR T cells-mediated anti-tumor immunity (Haynes et al., 2001), we decided to remove the non-functional, CD20-CD28-FceR1γ construct from further experimentations.

For the generation of a new superCAR construct we chose to incorporate the CD20-4-1BB-ζ into the pre-existing lentiviral expression vector pWPXLd carrying CD19-CD28-ζ (Figure 9) Due to the financial limits, we opted to implement this strategy by using general approaches of molecular cloning. The cloning of the CD20-4-1BB-ζ out of the lentiviral expression vector was complicated by the fact that several necessary sequences needed to be added to the 3´end of the construct itself. In order to be able to discriminate the individual CAR protein products, we intended to add a CAR-specific tag sequence detectable by a relevant cognate antibody. Further on, to ensure the monocistronic transcription of the two CARs from one open reading frame, we needed to separate the two constructs by the T2A sequence (Szymczak et al., 2004). Lastly, we planned to ligate the CD20-4-1BB-ζ into CD19-CD28-ζ/pWPXLd through the complementary restriction sites BglII/BamHI. CD19-CD28-ζ already contained a BamHI restriction site so we only needed to add the BglII site to both the 5´ and 3´end

of CD20-4-1BB-ζ (Figure 9). These conditions however led to the fact that the revers primer PO20-R for cloning of CD20-4-1BB-ζ had over 130bp and was causing significant technical difficulties (Table 5). The primer annealed non-specifically to the 5´end of the construct and upon amplification generated undesirable mutations. We attempted to introduce several optimizing steps to the PCR protocol, including various annealing temperatures and dividing the PCR reaction into two successive steps (Figure 12) as well as optimize the primer sequence, Unfortunately, sequencing of the final PCR product always revealed point mutations or larger insertions. These technical difficulties precluded us to complete the construction of the superCAR in time allocated for completion of this diploma thesis. It is clear that more time, effort and continuous optimization of this approach are needed to generate sequentially faithful construct.

Similar experimental approaches adapting simultaneous expression of two CAR receptors in one effector T cell have already been used. Notably, the offset of antigen escape by T-cells co-targeting HER2 and IL-13Rα2 in glioblastoma and enhanced antitumor activity was reported by Ahmed and his group (Hegde et al., 2013). Another approach is parallel targeting of two tumor antigens such as MUC-1 and the prostate stem cell antigen which are highly co-expressed in a variety of solid tumors by two distinct CAR T-cells lines mixed together. Such approach generates superior antitumor effect, however, it was reported that it is not enough to achieve a complete response (Anurathapan et al., 2014). Alternative approaches and strategies might be used in the future to generate the superCAR construct. Inspiration might come from a recently published approach adapted by Chen and her team. Instead of working with two separate CARs, they engineered a dual-antigen recognition construct built into a single-chain CAR molecule (Zah et al., 2016). This minimalistic system has several advantages. Due to its reduced size, such CAR construct exhibits increased transduction efficiency (Bos et al., 2010). Moreover production of bispecific single chain CARs is more cost effective compared to producing two distinct CAR T-cell lines whereby, in addition, CD19 specific CAR T-cells have the tendency to overgrow CD20 specific CAR T-cells despite the presence of the CD20 antigen (Zah et al., 2016). Strategies dealing with potential solutions for tumor antigen escape and overall functional regulation of CAR T cells are currently a hot topic in the field of CAR cancer therapy.

For gene transfer of CAR constructs into primary cells most frequently used approaches are retroviral or lentiviral transduction protocols (Sakuma et al., 2012; Wu et al., 2015). For the generation of CAR expressing Jurkat T-cell lines we decided to generate lentiviral particles (Kutner et al., 2009) (Figure 14) which are, unlike retroviruses, also efficient

for transducing non-dividing cells (Lewis et al., 1992). This could be useful for future experiments with patients T-cells. In our hands, the transduction efficiency of individual CAR constructs in Jurkat T-cell was below 20% (Figure 15) which is substantially less than reported in literature, where this efficiency in T cells reached up to 99% (Wu et al., 2015). Protocols differ in several points. The transgene expression vector size (Bos et al., 2010), the type of viral packaging plasmid (Cronin et al., 2005), the transfection reagent, and the resulting virus titers, all influence the final transgene expression efficiency (Cockrell and Kafri, 2007). It has also been reported that low expression of CD3 may be associated with the relatively low lentiviral transduction efficiency (Wherry, 2011). For technical reasons we did not determined titers of the viral particles and therefor it is likely, that virus concentration in our experiments were not optimal. Nevertheless, for functional studies, we sorted CAR expressing Jurkat T cells, so further experiments were done with a homogenously CAR positive cell populations.

CAR function was assessed by quantification of the T-cell activation-induced surface marker CD69. Its increased surface expression occurs within few hours after T-cell activation and this assay is therefore used as a standard method for monitoring T-cell activation (Simms and Ellis, 1996). We co-cultivated target $CD19^{+}CD20^{+}$ Raji cells (Figure 16) with CAR expressing T-cells analogously to the experiment presented by Wu and his colleagues (Wu et al., 2015), with altered co-cultivation ratio. To ensure maximum antigen source we increased the effector:target cell ratio from 1:2 to 1:4. We observed increased fraction of $CD69^{high}$ cells after overnight co-cultivation of Raji with CAR-expressing Jurkat cells. 26%, 13% and 16% of $CD3^{+}$ cells upregulated CD69 expression after co-cultivation with CD19-CD28-ζ, CD19-4-1BB-ζ and CD20-4-1BB-ζ, respectively (Figure 17). We can therefore conclude that individual CAR constructs are expressed on the surface of Jurkat cells and are functional as the recognition of their cognate antigen CD19 and CD20 on target Raji cells leads to their activation.

To confirm the functionality of individual CAR constructs by an alternative approach, we decided to try and take advantage of the mouse thymus lymphoma BW5147 cell line available in our laboratory which has the transcription factor NFAT fused with GFP. A similar experiment examining NFAT-dependent GFP expression in CAR expressing Jurkat cells was also conducted by Wu and his colleagues (Wu et al., 2015). We were not sure if the above mentioned experiment published by Wu et al. would be applicable to our conditions where we used instead of the human Jurkat cell a mouse cell line transduced with CAR constructs consisting of relevant human sequences. Our experiment was therefore an exploratory, proof of principle experiment. To minimize work with biohazard lentivirus we opted for safer transfection methods, at least initially when the result was uncertain. For unknown reasons

the transfection efficiency of BW5147 NFAT-GFP cell line was in general very low (Figure 18a). To obtain at least a minimal number of required CAR expressing cells, we had to use electroporation over lipofection. 12%, 13% and 6% of cells were CAR^+ for CD19-CD28-ζ, CD19-4-1BB-ζ and CD20-4-1BB-ζ, respectively after electroporation (Figure 18a). Even though we optimized the voltage, cell suspension concentration and DNA concentration, the efficiency was lower than expected so we decided to sort CAR^+ cells before co-cultivation experiments. Unfortunately, low electroporation efficiency resulted in very low numbers of sorted CAR^+ NFAT-GFP cells. Nevertheless we adjusted the cell ratios, established co-cultivation experiments and observed that a small but distinct population of CAR^+ cells was in fact GFP^+ after recognizing the CD19 or CD20 antigen on target Raji cells. 4.4%, 2.3% and 5.6% for CD19-CD28-ζ, CD1-4-1BB-ζ and CD20-4-1BB-ζ respectively were detected as GFP^+ (Figure 18b and c). The quality of this data is of course partly hampered by a low number of used cells and the results must be interpreted with caution. Despite this caveat, we consider the result as a proof of principle for validation of this system to test the efficiency of CAR construct. In the future however, it would be necessary to prepare a NFAT-dependent GFP expressing human cell line and use lentivirus for higher efficiency of transgene expression.

Because we were not able to prepare a functional superCAR construct that would encode for two distinct CD19-CD28-ζ and CD20-4-1BB-ζ polypeptide chains, we decided to try at least co-transfection of the two individual constructs into the NFAT-GFP mouse cell line and perform the co-cultivation experiment (Figure 19). There was no significant difference in NFAT-dependent GFP expression in cells electroporated by a single CAR construct and cells electroporated by both CAR constructs simultaneously. However, we could not distinguish whether both constructs are co-expressed as both CD19-CD28-ζ and CD20-4-1BB-ζ molecules have incorporated a myc-tag sequence. During the superCAR construction, we took this fact into account and tried to incorporate a distinct flag-tag sequence for the CAR CD20-4-1BB-ζ (Figure 9). This significantly contributed to the size and complexity of the reverse primer used for the preparation of CD20-4-1BB-ζ construct and likely contributed to the failure of superCAR cloning strategy. However, individual CAR constructs can be readily expressed and therefore we can assume that they may be co-expressed simultaneously by single cells (Hegde et al., 2013) and/or by a cell population (Anurathapan et al., 2014). These strategies could be also adapted for CAR co-expression. However, as mentioned above, the amount of DNA affects the transfection efficiency (Bos et al., 2010) and CD19 CAR expressing cells have the tendency to overgrow CD20 CAR expressing cells (Zah et al., 2016). Thus our experiment, with its inhere reported result, may only be considered as a pilot experiment out of which no final conclusions can be made.

Recently, several studies comparing the effect of CD28 and 4-1BB co-stimulation on CAR T-cell function, persistence and tumor eradication have been published (Kawalekar et al., 2016; Zhang et al., 2015; Zhao et al., 2015). Initially we also wanted to focus on the comparison of individual constructs with different co-stimulatory modules in relation to their cytotoxic activity. However, because so far we managed only initial experiments on the human Jurkat T-cell line and not on primary T-cells, theses comparative experiments are still ahead of us.

The overarching goal of this work was, in close collaboration with Dr.Otáhal, to contribute in building a new type of dual specificity CAR constructs for their implementation in CAR T-cell therapy for hematological malignancies at Czech clinics. The original idea was to construct a previously designed functional superCAR, compare its function with individual single CARs in cell lines, test its efficiency on an animal model and primary human cells. Very soon however, it became evident that this ambitious project would require more time, effort, skills and financial support, which go beyond the scope of work of one master student. The preparation of the individual constructs themselves was the most time consuming part and unfortunately we ran out of time to complete the construction of the new superCAR by classical methods of molecular cloning. The essential part of this work comparing individual single CAR constructs with the superCAR construct could not therefore be accomplished. Nevertheless, I see tremendous benefits from working on this exciting project. This work laid the groundwork for future experiments in a very perspective field of immunotherapy that is yet to be implemented in the Czech clinic. On top of that, this work has taught me a great deal of laboratory techniques, methods and protocols that are widely applicable for many research fields of experimental biology, in general, and immunology, in particular., and which are indispensable for my future work in science.

6. Conclusion

The overarching goal of this work was to prepare and evaluate recombinant constructs for their possible implementation in CAR T-cell therapy for hematological malignancies into the Czech clinic. This was a collaborative effort with MUDr. Pavel Otáhal, the pioneer of CAR technology in Czech Republic, with specific goals to generate functional CAR constructs specific for the B-cell lineage antigens CD19 and CD20 and ultimately to generate a functional superCAR that would endow T-cells with the dual specificity for both antigens CD19 and CD20.

We demonstrated that individual CAR constructs, built up from distinct segments of several co-stimulatory molecules were fully functional and worked independently of each other. Jurkat T-cell lines expressing individual CD19 and CD20 CAR constructs were successfully generated by lentiviral transduction and their targeting capacity was confirmed by two alternative approaches. The generation of a new functional superCAR construct was initiated, however, it was not accomplished in time dedicated to complete the work on this thesis. This was apparently due to a complicated cloning strategy and several technical difficulties that slowed down the progress of our work. Thus, because the generation of the superCAR construct was not yet completed, we were not able to compare the efficiency of individual single CAR constructs with that of superCAR. It is of note that such comparison would be possible only in real physiological conditions, i.e. using an appropriate animal model or human patients, where the frequency of malignant B-cell clones able to escape the detection upon losing one of the markers, CD19 or CD20, can be assessed.

Thus, although the ultimate objective of this ambitious project was not accomplished within the realm of this thesis, we have acquired the methodology and set the groundwork for future experiments. Employment of CAR Jurkat T-cell lines in functional experiments were extremely educative and enabled to acquire necessary laboratory skills and expertise which are essential and indispensable for future scientific endeavors in this fast progressing field of immunotherapy.

The CAR technology for cancer immunotherapy is a perspective therapeutic strategy worth of research effort in the Czech Republic. Future experiments would focus on finishing the generation of the superCAR construct and testing new CAR constructs in primary human T-cells, in animal models and in the future, perhaps also in oncological patients. Although, given the local conditions and expertise, it is extremely difficult to compete with large American and Asian centers specializing on CAR immunotherapy, we are very hopeful that our

work in cooperation with MUDr. Pavel Otáhal could bring this therapeutic technology closer to patients in the Czech Republic.

7. Appendix

Center	Malignancy	co-stimualtion	Gene modification	Disease status	Conditioning therapy	ClinicalTrials.gov identifier
Shenzhen Second People's Hospital	CLL ALL Lymphoma	CD28-CD3ζ	retrovirus/ retrivirus	relapse/ refractory	CF	NCT02456350
National Cancer Institute	B-cell lymphoma B-cell leukemia HL, NHL		retrovirus	14 days from last treatment	CF	NCT02659943
National Cancer Institute	Pediatric/adolescent B-ALL or lymphoma	CD28-CD3ζ	retrovirus	relapse/ refractory	at discretion of investigator	NCT01593696
National Cancer Institute	any	CD28-CD3ζ	retrovirus	relapse/ refractory	CF	NCT00924326
Abramson Cancer Center of the University of Pennsylvania	CLL SLL			relapse/ refractory	*Ibrutinib ?	NCT02640209
Abramson Cancer Center of the University of Pennsylvania	ALL	4-1BB-CD3ζ	lentivirus	relapse/ refractory		NCT02030847
Abramson Cancer Center of the University of Pennsylvania	adult CLL/SLL	4-1BB-CD3ζ		relapse/ refractory		NCT01747486
Abramson Cancer Center of the University of Pennsylvania	HL	4-1BB-CD3ζ	electroporation	relapse/ refractory		NCT02277522
Abramson Cancer Center of the University of Pennsylvania	Lymphoma	4-1BB-CD3ζ		relapse/ refractory		NCT02030834
Abramson Cancer Center of the University of Pennsylvania	MCL Diffused large B-cell lymphoma Follicular lymphoma			relapse/ refractory	Pembrolizumab	NCT02650999
University of Pennsylvania	children B-cell lymphoma B-cell leukemia	4-1BB-CD3ζ	lentivirus	chemo-resistante refractory	variable chemotherapy	NCT01626495
University of Pennsylvania	HL	4-1BB-CD3ζ	electroporation	relapse/ refractory		NCT02624258

Jichi Medical University	NHL		retrovirus	relapse/ refractory		NCT02134262
Kite Pharma, Inc.	DLBCL PMBCL TFL			relapse/ refractory	CF	NCT02348216
Kite Pharma, Inc.	B-precursor ALL			relapse/ refractory		NCT02614066
Kite Pharma, Inc.	pediatric/adolescent B-precursor ALL			relapse/ refractory		NCT02625480
Kite Pharma, Inc.	MCL			relapse/ refractory	C	NCT02601313
Beijing Doing Biomedical Co., Ltd.	ALL, CLL, NHL			relapse/ refractory		NCT02656147
Beijing Doing Biomedical Co., Ltd.	ALL, CLL, NHL			relapse/ refractory		NCT02546739
Shanghai GeneChem Co., Ltd.	B-cell leukemia	4-1BB-CD3ζ		relapse/ refractory		NCT02672501
Southwest Hospital, China	ALL, CLL, NHL			relapse/ refractory		NCT02349698
Fred Hutchinson Cancer Research Center	ALL, CLL, NHL	4-1BB-CD3ζ	lentivirus	relapse/ refractory		NCT01865617
Fred Hutchinson Cancer Research Center	NHL	4-1BB-CD3ζ	lentivirus	relapse/ refractory	*Durvalumab (mAb against PD-L1) C	NCT02706405
Peking University Cancer Hospital	B-cell lymphoma	CD27-CD3ζ	lentivirus	relapse/ refractory		NCT02247609
Chinese PLA General Hospital	B-cell leukemia B-cell lymphoma	4-1BB-CD3ζ	retrovirus	relapse/ refractory		NCT01864889
Chinese PLA General Hospital	MCL	4-1BB-CD3ζ		relapse/ refractory		NCT02081937
Shanghai Tongji Hospital, Tongji University School of Medicine	B-cell lymphoma B-cell leukemia		lentivirus	relapse/ refractory		NCT02537977
Xinqiao Hospital of Chongqing	B-cell lymphoma	CD28-CD3ζ	retrovirus	relapse/ refractory		NCT02652910
Baylor College of Medicine	NHL ALL CLL	CD28-CD3ζ + CD28-4-1BB-CD3ζ	retrovirus	relapse/ refractory	C	NCT01853631
Baylor College of Medicine	NHL ALL CLL	CD28-CD3ζ / virus specific T-cells	γ-retrovirus	relapse/ refractory	none	NCT00840853
Baylor College of Medicine	Lymphoma CLL, ALL	CD3ζ (+ CD28-CD3ζ)	γ-retrovirus	relapse/ refractory	Ipilimumab or none	NCT00586391
Baylor College of Medicine	NHL ALL CLL	CD28-CD3ζ	γ-retrovirus	relapse post allo HSCT	none	NCT02050347

Baylor College of Medicine	NHL CLL	EBV-specific CAR T-cells +CD28-CD3ζ	γ-retrovirus	relapse/ refractory	C or none	NCT00709033
Memorial Sloan Kettering Cancer Center	CLL indolent lymphoma	CD28-CD3ζ +4-1BB-CD3ζ	γ-retrovirus or lentivirus	relapse/ refractory	C	NCT00466531
Memorial Sloan Kettering Cancer Center	pediatric ALL	EBV-specific CAR T-cells +CD28-CD3ζ	γ-retrovirus	relapse/ refractory	variable chemotherapy	NCT01430390
Memorial Sloan Kettering Cancer Center	CLL	CD28-CD3ζ	γ-retrovirus	relapse/ refractory	C	NCT01416974
Memorial Sloan Kettering Cancer Center	adult ALL	CD28-CD3ζ	γ-retrovirus	relapse/ refractory	C	NCT01044069
Memorial Sloan Kettering Cancer Center	NHL	CD28-CD3ζ	γ-retrovirus	relapse/ refractory	chemotherapy	NCT01840566
Memorial Sloan Kettering Cancer Center	pediatric and young adult ALL	CD28-CD3ζ		relapse/ refractory	C	NCT01860937
City of Hope Medical Center	intermediate-grade B cell lymphoma	no costimulation	lentivirus	relapse/ refractory	APBSCT	NCT01318317
City of Hope Medical Center	ALL	CD28-CD3ζ	lentivirus	relapse/ refractory	chemotherapy	NCT02146924
M.D. Anderson Cancer Center	B-cell lymphoma CLL	CD28-CD3ζ	transposon	relapse/ refractory	APBSCT	NCT00968760
M.D. Anderson Cancer Center	prophylaxis or active malignancies	CD28-CD3ζ	transposon	relapse/ refractory	None	NCT01497184
M.D. Anderson Cancer Center	prophylaxis or active malignancies	CD28-CD3ζ	transposon		None	NCT01362452
Uppsala University	B-cell lymphoma B-cell leukemia	CD28-4-1BB-CD3ζ	γ-retrovirus	relapse/ refractory		NCT02132624
Second Military Medical University	B-cell lymphoma B-cell leukemia			relapse/ refractory	CF	NCT02644655
The Second Affiliated Hospital of Henan University of Traditional Chinese Medicine	B-cell lymphoma B-cell leukemia	CD28-CD3ζ +4-1BB-CD3ζ		relapse/ refractory		NCT02685670
Juno Therapeutics, Inc.	ALL	CD28-CD3ζ	virus	relapse/ refractory	chemotherapy	NCT02535364
Juno Therapeutics, Inc.	NHL MCL DLBCL			relapse/ refractory	chemotherpay	NCT02631044
Seattle Children's Hospital	child and young adult B-cell leukemia		lentivirus	relapse/ refractory		NCT01683279
University College, London	DLBCL			relapse/ refractory	CF	NCT02431988

University College, London	pediatric and young adult ALL Burkit lymphoma			relapse/ refractory	CF	NCT02443831
Novartis Pharmaceuticals	DLBCL		lentivirus	relapse/ refractory		NCT02445248

Table 15: Ongoing and recruiting CAR19 clinical trials.

Information summarizing ongoing and recruiting clinical trials using CAR T-cells for immunotherapy of hematological B-cell malignancies were retrieved from clinicaltrials.gov.

The following abbreviations stand for: ***CLL****-chronic lymphocytic leukemia;* ***ALL****-acute lymphoblastic leukemia;* ***SLL****-small lymphocytic leukemia;* ***HL****-Hodgkin´s lymphoma;* ***NHL****-Non-Hodgkin´s lymphoma;* ***MCL****- mantle-cell lymphoma;* ***DLBCL****- Diffuse large B-cell lymphoma;* ***PMBCL****- Primary mediastinal B-cell lymphoma;* ***TFL****- Transformed follicular lymphoma; C-cyclophosphamide; F-Fludarabine;* ***HSCT****-hematopoietic stem-cell transplant;* ***APBSCT****-Autologous peripheral blood stem cell transplantation*

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