TOWARDS SAFE AND EFFECTIVE CD38-CAR T CELL THERAPY FOR MYELOMA

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TOWARDS SAFE AND EFFECTIVE CD38-CAR T CELL THERAPY FOR MYELOMA

OP WEG NAAR EEN VEILIGE EN EFFECTIEVE CD38-CAR T CEL THERAPIE VOOR MYELOOM

(met een samenvatting in het Nederlands)

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CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The rise of immunotherapy

Our immune system has the ability to protect the body from foreign pathogens (viruses, fungi and bacteria) as well as developing tumors. However the immune system can fail to control the outgrowth of malignant cells and tumors will develop. Conventional cancer therapies consist of surgery, radiotherapy, chemotherapy or a combination of these modalities. Even though these therapies can be successful, there is an ongoing development of more effective and less toxic treatment options. Many of these new approaches focus on a more tumor-specific strategy using antigen-specific features of the immune system. One of the most promising antigen-specific immunotherapies is antibody therapy, where patients are treated with tumor-reactive antibodies. While some antibodies have been developed to block essential functions of tumors[1–4], many antibodies are administered either to directly induce the apoptosis of the tumors[5–7] or to enable tumor cell recognition and subsequent killing by immune effector cells that express FC gamma receptors, such as natural killer (NK) cells, neutrophils and mononuclear phagocytes[8–11]. Antibody therapy can be very effective, but it is a passive immunization strategy, as there is no active antibody production by the patient's immune system. Immunotherapy approaches based on another antigen-specific component of our immune system, such as T cells, provide an active 'living' therapy, creating an immune memory.

T cell-based immunotherapy

A T cell immune response can be induced by recognition of a foreign peptide antigen presented by a major histocompatibility complex (MHC) molecule at the cell surface. Virtually all antigenic peptides of tumors are generated inside the cell by enzymatic cleavage of intracellular proteins[12]. In many cancers, mutations in the tumor's genome can give rise to intracellular proteins that are slightly different from the original protein sequences. Peptides derived from the altered regions of these mutated proteins can bind to MHC molecules and are presented to the T cells as foreign antigens[13,14]. These so-called neo-antigens, which sometimes arise even from a single nucleotide mutation, can evoke a powerful T cell response that is ultimately able to complete eradicate the tumor[15–19]. Nonetheless, in many cases the anti-tumor immunity is insufficient to effectively control the tumor growth, either due to complex tumor-escape mechanisms, such as the downregulation of the MHC molecules, or due to a heavily tumor-protective microenvironment leading to the suppression or exhaustion of tumor-reactive T cells[20–23].

To improve T cell efficacy and increase their effector function, the very first immunotherapy method was to administer the T cell growth factor IL-2[24]. Addition of this cytokine to T cells in *in vitro* cultures as well as in mice lead to a significant tumor reduction, which was later confirmed in patients suffering from metastatic melanoma[25]. Also, the tumor infiltrating lymphocytes (TILs) isolated from patient's melanoma dissections, could be expanded *ex vivo* and their killer functions were improved by addition of IL-2. This suggested that these

revitalized TILs could be used to treat patients[26,27]. Reinfusion of TILs, indeed, resulted in significant tumor regression in melanoma patients[28]. While TIL therapy should be in principle possible for many types of tumors, the most successful TIL studies have been conducted in the setting of melanoma, which is an example of an aggressive, but highly immunogenic cancer type because its fast growth rate induces a high mutational load, leading to many immunogenic tumor antigens[13].

Dendritic cell-based immunotherapy

Whereas TILs represent the patients' normally occurring T cell response to the tumor, several clinical studies have also demonstrated the possibility to prime the T cells of patients against tumors by vaccination with tumor antigen-loaded dendritic cells (DCs), the most superior antigen presenting cells of our immune system. There are currently several ways of antigen loading of these *ex vivo* generated DCs, including DNA or RNA of the tumor-antigen, incubation with tumor lysate, proteins and/or with (mixtures) of peptides[29–34], or even fusing tumors with DCs[35,36]. More recently, oncolytic vaccines have been used to deliver tumor genetic information to DCs *in vivo*.[33] An alternative strategy to *in vivo* load DCs with tumor antigens is vaccinating the patients with antigens coupled to DC-specific ligands[37,38] or antibodies[39]. Overall, DC vaccines have not yet revealed their clinical potential, since many important requirements for inducing a long-lasting T cell immune response by DCs are not entirely understood[40]. Nonetheless, there are several attempts to enhance the effect of DC vaccination strategies, either by combinations with immunomodulatory agents or by determining the optimal (cytokine) adjuvants[34,41].

Generating tumor-specific T cells

Alternative to the priming of T cells by DC vaccinations, another treatment option is the use of *ex vivo* generated tumor-specific T cells. The initial studies of such adoptive immunotherapy often used repeated stimulations of patients' T cells with antigen loaded DCs to generate the tumor-reactive T cells. Although this strategy is still highly effective to generate for instance virus-specific T cells to treat virus associated tumors[42], in the recent years the most frequently applied method is to introduce a tumor-specific T cell receptor in the patients' T cells. Therefore often retroviral-[43], lentiviral-[44] or transposon-based[45] gene delivery systems are used to integrate the TCR gene in the T cell's genomic DNA. Generating a transient TCR expression is also possible after electroporation with mRNA constructs[46]. The field of controlled and specific genetic manipulation is currently expanding significantly with the platforms such as Transcription Activator-Like Effector Nucleases (TALEN) or the newest break-through: Clustered Regularly Interspaced Short Palindromic Repeats-Cas9 (CRISPR-Cas9)[47,48].

Immune checkpoint blockade

Even if T cells are present, they can be significantly suppressed by the tumor microenvironment. Therefore, next to the development of strategies to induce and expand T cells, recent studies also aimed to remove the 'breaks on T cells', by administrating antibodies which block T cell inhibitory molecules (immune checkpoints) such as programmed cell death-1 (PD-1)[49], its ligand PD-L1[50] or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)[51]. The clinical responses to these checkpoint blockade appeared very successful and often durable[49,52,53]. It was found that the clinical response were indeed associated with tumor mutational load[54–56], indicating that an genetically unstable tumor will elicit a more abundant T cell response.

The immunotherapy options described until here, nicely illustrate several possibilities to exploit T cell immune functions to actively control tumor growth. Nonetheless, as mentioned, many of these strategies require the presence or the generation of sufficient anti-tumor T cell responses. Nevertheless, there are several tumor types which are not associated with a high mutational load and are therefore incapable of inducing sufficient anti-tumor T cell responses. Due to poor tumor antigenicity, DC vaccinations may also be less effective in these cases. If a good T cell response is not present or cannot be generated, immunotherapy strategies to boost T cells, such as the immune checkpoint blockade, may not be effective. To efficiently target these tumors, other strategies need to be considered.

CHIMERIC ANTIGEN RECEPTORS

The rise of genetic engineering resulted in numerous possibilities to design more innovative approaches to target the antigen-specific immune system towards tumors. One of the most promising approaches of today is the Chimeric Antigen Receptor (CAR), by which the superior antigen tracing capacities of antibodies are coupled with the superior killer mechanisms of T cells. Initially, this idea started with the development of the first fusion proteins consisting of the antibody-derived single chain variable fragments (scFv) coupled to the TCR alpha and beta chains[57]. Variation to this theme resulted in the engineering of functional T cell fusion proteins targeting CD4[58], CD8 or cytokine receptors[59] and consisted of the TCR-CD3 complex derived CD3ζ-chain[60].

Eventually the first CARs were developed, the so-called 'T-bodies'. These chimeric molecules consisted of an antibody recognition domain coupled with the T cell activating CD3ζ domain[61]. The T cells genetically engineered to express these CARs were functional but became rapidly anergic, since the CAR generated only the first T cell activation signal through the CD3ζ chain[62–64]. For full T cell activation, it appeared that next to TCR-signal, additional signals were needed to further stabilize the T cell activation. In conventional T cells such "costimulatory" signals are generated by the interaction of surface molecule CD28 with CD80 (B7-1) or CD86 (B7-2) on antigen presenting cells. Secondary interactions include proteins of the TNF receptor family such as 4-1BB with 4-1BB-ligand (4-1BBL)

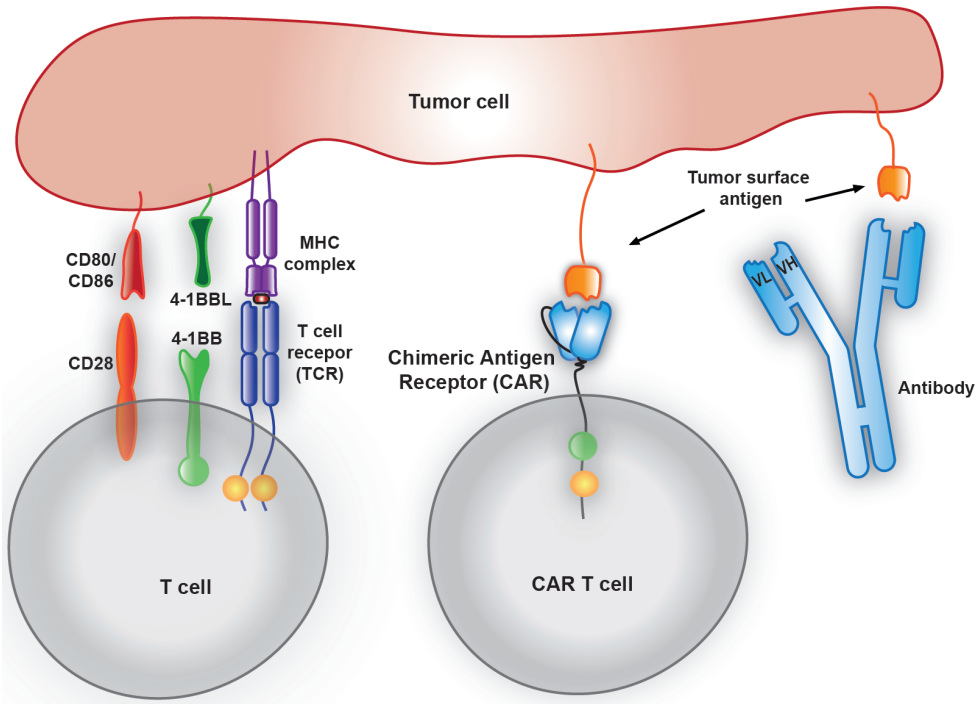

Figure 1. Schematic overview of Chimeric Antigen Receptor T cells. At the left side the conventional T cell expressing a T cell receptor (TCR) which recognizes a peptide presented in a major histocompatibility complex (MHC). Activation of a T cell is further aided by the co-expression of costimulatory molecules such as CD28 and 4-1BB.On the right side an antibody which recognizes the intact tumor surface antigen with variable sites of the antibody, the variable heavy chain (VH) and the variable light chain (VL). In the middle, the CAR, with the VH and VL of the antibody, and transmembrane and intracellular domains of the TCR and, in this case the costimulatory protein 4-1BB.

and OX40 with OX40-ligand, which can further stimulate T cells[65–68]. Indeed, addition of costimulatory domains CD28 and 4-1BB to the CAR construct allowed T cell to be fully activated, to optimally expand and to persist *in vivo* after administration to patients[69,70]. These CAR configurations are generally referred to as "second generation" CARs (see figure 1). Many comparisons and variations to this second generation CAR design were investigated recently. The general consensus from these studies is that CD28 harboring CARs are using the fast-acting AKT pathway[71], leading to rapid T cell responses but faster exhaustion[72]. On the other hand CARs with 4-1BB use the NF-kB pathway, are more persistent, preserve a central memory phenotype[73] and produce more cytokines like IFN-γ[74]. To enhance the second generation CARs by introducing 2 signaling domains into the same CAR, "third generation CARs", did not result in significant clinical benefit so far[75,76]. However, other designs, also described in this thesis, introduced signaling domains separate from the CAR which increased *in vivo* efficacy and persistence[65,77]. A timeline overview of important CAR T cell developments is given in figure 2.

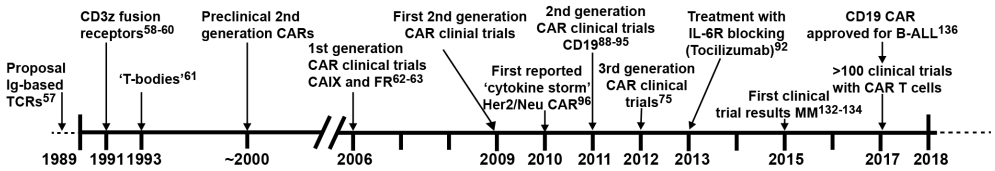

Figure 2. Timeline of important milestones in CAR T cell development. (references are indicated in superscript)

Advantages of Chimeric Antigen Receptors compared to conventional T cell therapies

Many of the recent research is focused on the improvement of CAR therapy, as outlined below, there are several advantages of this therapy in comparison to conventional T cell therapy.

No MHC restriction

A major advantage of using CAR-engineered T cells over conventional T cells or TCR engineered T cells is the recognition of the tumor antigen in a major histocompatibility complex (MHC)-independent manner. Therefore, CAR therapies can be broadly applied without the need for selecting patients according to their MHC type. The MHC restriction significantly limits the broad application of conventional T cell therapies. Furthermore, since there is no MHC involved in tumor recognition, CAR therapies do not suffer from the downregulation of MHC, which can be an important mechanism of tumor escape in T cell therapies[66,78,79].

Recognition of intact proteins

In contrast to T cells, CAR technology allows targeting of many different antibody epitopes on intact surface molecules, based on the specificity of an antibody with the potent effector function of T cells. This extends the variety of targetable antigens from the peptide-restriction of T cells, towards mutated tumor associated proteins[80], alternatively spliced variants[81] or different glycosylation patterns[82,83]. Furthermore, in contrast to conventional T cells CAR T cells can target tumor associated non-protein antigens, for example the ganglioside GD2 to treat neuroblastoma[84].

No TCR mispairing

In case of T cell engineering with a complete TCR, the introduced α- and β-chain can pair with the α- and β-chain of the endogenous TCR as they compete for the same TCR-CD3 complex[85,86]. TCR mispairing could also lead to unknown (auto-)reactivity with potential cytotoxicity[87]. In case of CAR transduction, there is no competition for CD3 docking or TCR mispairing.

Strong anti-tumor responses

The CAR can induce a rapid and stringent T cell response, because both the recognition, activation and costimulation are aligned in one molecule to fully activate the T cell. Since costimulatory signal is readily provided, CAR T cells are also unlikely to display anergy, which can be a problem in the conventional T cell therapies directed against tumors expressing low level of costimulatory ligands.

Currently, there are almost 200 clinical trials using CAR T cells. More than half of these studies are targeting CD19, a B cell specific marker. The impressive results obtained in several studies in B-cell leukemia and lymphoma, even when in heavily pre-treated, chemotherapy resistant patients, illustrated the power of CAR T cells in the battle against hematological tumors[69,88–92]. Nonetheless, the field of CAR T cell therapy is still evolving and expanding. The clinical experience with CAR T cells have also identified several drawbacks of CAR T cell therapy that need to be tackled in order to make this type of therapy safer and suitable for more patients.

Drawbacks of Chimeric Antigen receptors

Although CAR therapy has many advantages, it has also disadvantages and drawbacks. The main disadvantage of CAR therapies is the inability to target intracellular tumor antigens, several of which are (intracellular) mutated genes, thus neo-antigens. Furthermore, there are a number of important issues to be addressed. These current drawbacks of CAR therapy can be roughly dissected into those related to the on-target and off-tumor effects.

Drawbacks due to on-target effects

On-target toxicity occurs when the antigen recognition induces an excessive T cell response. T cells and other immune cells start to increase their activation and release of cytokines, often IL-6 and IFN-γ[91]. This could lead to a cytokine storm, or cytokine release syndrome (CRS) which is characterized by fever, nausea and headache but can also lead to more severe, sometimes even, fatal side effects like respiratory insufficiency, hypotension, and multi-organ failure[93,94]. In addition, pancytopenia such as thrombocytopenia and neurological effects including epileptic insults have been described[89–92,95,96]. Several fatalities have been reported, which in some cases resulted in discontinuation of the clinical study. Also, as described by Porter *et al.*[69] the severity of CRS was associated with tumor burden[91]. Therefore, reduction of tumor load is suggested as important pretreatment prior to CAR T cell infusion[97].

Initially, anti-inflammatory corticosteroids were used to treat CRS, however this resulted in the complete eradication of CAR T cells. Later, a targeted form of CRS treatment with anti-IL6 receptor antibody (Tocilizumab) was successful[94]. When using this blocking antibody, most of the symptoms of CRS disappeared within a few hours[98].

Drawbacks due to off-tumor effects

Off-tumor effects include the cytotoxicity and response resulting from recognition of the correct target on non-tumor cells. Even the most successful CAR studies experience these toxicities because there are so far only a few truly tumor-specific antigens can be targeted by CAR T cells. The often targeted antigen CD19 is expressed on all B cells, leading to a complete eradication of the B cell compartment. Fortunately, this is not lethal to the patient and lack of B cells can be compensated by monthly immunoglobulin injections. However, for other cancer types there often is no tissue exclusive targetable molecule. To further develop CAR T cell therapy, improving safety is imperative. Several approaches have emerged that can control CARs. These approaches can be roughly divided into two categories: a) strategies improving the specificity of CARs or b) controlling the CAR activity (Table 2).

Improving safety of CARs

Preventing off-tumor cytotoxicity

The safest option would be to target only tumor-specific antigens. Unfortunately, oncological targets often share expression with healthy tissue. Currently, there are only a few genuinely tumor-specific antigens that can be targeted by CAR T cells, which include the spliced variant of the epidermal growth factor receptor (EGFR), isoform VIII, which was found to be specifically expressed on glioblastoma cells and other solid tumors such as breast cancer in a fraction of patients and resulted in accelerated approval for clinical testing[99]. Also the variant isoform of the commonly expressed CD44, isoform 6 (CD44v6) seems to be expressed in a cancer-specific fashion in certain patients. CAR T cells directed towards CD44v6 were tested in a pre-clinical model[80]. There are only a few examples, all other antigens targeted by CAR T cells are either lineage specific antigens or antigens that are overexpressed in cancer tissue.

Table 1. Approaches to enhance CAR safety

Preventing Off-tumor cytotoxicity	Safer antigen	Tumor specific Mutated or glycosylated targets
	Sensitivity of scFv	Tuning affinity
	Combinatorial design	Dual CAR Syn-Notch
Preventing (too strong) On-target cytotoxicity	Inducible CAR	iCAR
	Suicide gene	HSV-TK, iCasp9 and mAb targets
	Coupling of CAR/masked CAR	

Optimizing the affinity

The overexpression of antigens on tumor cells can be exploited by CARs with diminished affinity. These attenuated CARs only respond to a dense antigen expression on tumor cells, without harming lower expressing cells, thereby preventing on target/off tumor effects[100–102]. This opens a therapeutic window in which antigen expression on healthy cells is too low to activate CAR T cells while tumor cells are highly susceptible. CARs with diminished antigen affinity have been successfully engineered for ErbB2, EGFR, ROR1[99-102] and in our studies for CD38, which is described in this thesis.

Combinatorial design

Dual CAR designs aim to increase tumor specificity by providing T cells with two CARs, each with a different antigen specificity and each providing one of the two T cell activation signals. The requirement here is that the tumor expresses both antigens simultaneously, while normal tissue does not. For instance, when a tumor cell expresses antigen A and B, and the healthy tissue expresses only A *or* B, CARs targeting A and B are only fully activated when both receptors recognize their target at the same time[103–106]. Healthy tissue expressing either one of the targets cannot fully activate the T cells and will be protected. Vice versa, T cell activation can be shut down in the presence of healthy cells. Such inhibitory or "iCARs" couple a scFv, specific for a healthy tissue antigen, to an inhibitory domain from for example PD-1 or CTLA4. This way, antigens on healthy cells will inhibit T cell activation and are thereby protected.

Another design of a combinatorial antigen includes usage of the synthetic Notch receptor platform (synNotch). These artificial receptors contain an extracellular recognition domain and an intracellular transcription-activating domain[107]. Roybal *et al.*[108] demonstrated upon recognition of the first antigen, the synNotch receptor is cleaved and regulates transcription of the second, cytotoxic CAR.

Preventing on-target cytotoxicity – *in vivo* T cell control

Suicide genes

To avoid undesired or unexpected toxicities, engineered T cells can be armed with "suicide genes". The first described suicide gene is a thymidine kinase (TK) derived from herpes simplex virus (HSV-TK). T cells expressing HSV-TK are killed when this protein is converted into a toxic form by the anti-viral drug ganciclovir[109]. However, the HSV-TK protein itself can elicit an unwanted immune responses as it is virus-derived. Another suicidal system is based on the apoptosis machinery. During normal apoptosis, caspase 9 is dimerized and induces cell death. Genetically engineering of the caspase 9 structure, allows caspase dimerization to be induced by a small molecule (dimerizer)[110,111]. CAR T cells expressing this modified inducible caspase 9 (iCasp9) can then be specifically eliminated by administration of the dimerizer to the patient[112]. Another approach for the selective ablation of engineered cells can be achieved when they express (parts) of a surface

antigen such as truncated EGFR (tEGFR) or CD20. Cells expressing these markers are then eradicated upon infusion of monoclonal antibodies either Cetuximab (anti-EGFR) [113] or Rituximab (anti-CD20)[114].

Inducible CARs

In contrast to disabling CAR T cells, specific induction of CAR expression may provide an alternative opportunity to gain more control over these cells. As described in the Syn-Notch strategy the expression of a CAR can be regulated on transcriptional level. On-switches may be safer than off-switches or suicide genes since the default setting is "off". An example of such an on-switch system is the tetracyclin (Tet)-on system. In this system a cell is provided with a gene that transcribes the reverse tetracycline Transcriptional Activator (rtTa) protein, which binds to tetracycline response elements in the promoter region, only in the presence of doxycycline. This then activates transcription of a down-stream gene, for example a CAR[115,116]. It is important to study the specific kinetics of this on-switch system, to apply the correct dosage of doxycycline and to monitor the decay when doxycycline treatment is stopped. An important advantage of an on-switch is the ability to remove the inducing agent when adverse effects occur and to reintroduce it if tumor progression is observed.

In several recent CAR design developments, other switchable CARs have been proposed. For instance, the decoupling of the CAR's extracellular antigen-binding domain and the intracellular signaling domain. Upon application of a specific dimerizer (a rapamycin analog) the CAR can be (re-)assembled through an heterodimer protein interaction, the so-called FKBP-FRB module[117]. Similarly, the development of 'masked CARs' can delay CAR activity by masking the antigen binding site with a peptide, separated by a protease specific amino acid sequence[118]. Some tumor microenvironments contain a higher number of proteases, which can then cleave this peptide and enable site-specific CAR activation.

MULTIPLE MYELOMA

In this thesis, we specifically aimed at designing improved CAR T cell therapies for multiple myeloma (MM), this plasma cell malignancy, which accounts for 10% of hematologic malignancies worldwide is still incurable, although the treatment options expanded and survival rates have increased in the past 30 years. Especially, treatments with immunomodulatory drugs such as lenalidomide and proteasome inhibitors resulted in better clinical responses and improved patient survival. Recently, the treatment with antibodies, especially with the monoclonal antibody daratumumab has raised considerable optimism for antibody based immunotherapy. Daratumumab was not only found to be effective[119], but collateral damage to CD38-positive T regulatory cells appeared to be an advantage for an effective anti-tumor response[120], suggesting a T cell mediated component of daratumumab based immune therapy. Next to these achievements, the well-known graft-versus-tumor effects of allogeneic stem cell transplantation (SCT)

and donor lymphocyte infusions[121] and the long-term survival of several MM patients after SCT suggests that powerful T cell-based immunotherapeutic approaches could lead to effective anti-MM immunity. Therefore, we, similar to several investigators, hypothesized that the strong anti-tumor effects of CAR transduced immune cells could lead to significant responses in MM. In fact, there are currently many CAR strategies being developed for MM, which are summarized below.

Chimeric antigen receptors for MM

There are several MM associated antigens, which are considered for CAR T cell therapies. While some are in preclinical development, many of them, such as CD138, kappa light chain and the B cell maturation antigen (BCMA) have been tested in clinical trials (Table 2).

CD138-CAR

CD138 is a plasma cell-associated antigen expressed at high levels on MM cells. Interim results[122] of CD138-CAR T cell treatment showed that these cell infusions were well tolerable and four out of the five patients achieved stable disease. However, enhancing CD138-specific cells could lead to toxicity, as CD138 has shared expression on epithelial cells. Furthermore, CD138 is susceptible to immune escape as it is often downregulated on malignant cells[123].

Lewis Y-CAR

The Lewis Y antigen (Le^y) is expressed on epithelium but at a higher level in many epithelial malignancies as well as on acute myeloid leukemia and MM cells[124,125]. A CD28-based second-generation CAR was generated[126] and indicated a strong correlation between Le^y expression levels and CAR-induced cytotoxicity[124]. Clinical benefits as well as epithelium directed cytotoxicity are not yet determined.

Kappa light chain-CAR

Kappa light chain specific CARs would eliminate B cells that are not lambda but kappa light chain positive. Even though MM cells are often kappa-negative it is thought that the MM initiating cells can be eliminated[127]. Treatments with kappa-specific CARs, resulted in strong responses in non-Hodgkin lymphoma and B-cell acute lymphoblastic leukemia (B-ALL) patients. For MM patients, results were less stringent, the clinical trial resulted in 5 patients with stable disease (SD) and 2 non-responders[127].

BCMA-CAR

Currently there are several trials targeting BCMA[128,129], since gene expression profiling indicated a high expression of this molecule on plasma cells[130]. However, BCMA expression levels on tumor cells of different MM patients indicated a wide variability and often an even low cell surface expression[131]. Still, the number of trials targeting BCMA is expanding,

recent results from clinical trials showed more encouraging results with the majority of patients having at least a partial response[132–135]. This data suggested that the treatment of relapsed/refractory MM patients with BCMA-CAR T cells had the potential to induce durable responses. These finding led to an accelerated FDA approval to treat this patient population[136].

CD19-CAR

Interestingly, a CAR trial targeting CD19 also started to include MM patients. CD19 is not associated with MM, often not even expressed on most of the malignant cells. However there was a response seen in a MM patient treated with CD19-CAR T cells post auto-SCT[137], which led to the extended recruitment of MM patients in this study.

Table 2. Ongoing CAR T cell clinical trials for Multiple Myeloma.

Target antigen	Results	Ref	Institute	Status	NCT Number
CD19	1 CR, 6 VGPR, 2 PR, 2 PD	[137]	University of Pennsylvania USA	Ongoing	NCT02794246
CD138	4 SD, 1 PD	[122]	PLA General Hospital, China	Ongoing	NCT01886976
Lewis Y			Peter MacCallum, Australia	Ongoing	NCT01716364
NKG2D ligands			Dana Farber, Celyad, NHLBI, USA	Ongoing	NCT02203825
Kappa light chain	5 SD, 3 NR	[127]	Baylor, Texas, USA	Ongoing	NCT00881920
CS1			City of Hope, USA	Will open soon	
BCMA			Nanjing Legend Biotech Co. China	Ongoing	NCT03090659
BCMA			Xinqiao Hospital of Chongqing, China	Ongoing	NCT03093168
BCMA			Southwest Hospital, China	Ongoing	NCT02954445
BCMA			MSKCC/Juno, USA	Ongoing	NCT03070327
BCMA	1 CR, 2 VGPR, 1 PR, 8 SD	[132]	NIH-NCI, USA	Ongoing	NCT02215967
BCMA	1 CR, 1 VGPR, 1 SD, 1 MR, 1 PD	[133, 134]	University of Pennsylvania USA	Completed	NCT02546167
BCMA	1 CR, 1 VGPR, 4 PR, 1 SD, 1 PD	[135]	Bluebird Bio, USA	Completed	NCT02658929

CR=complete response, VGPR=very good partial response, PR=partial response, SD=stable disease, MR=minimal response, PD=progressive disease and NR=no response.

1

CAR T cells in preclinical development

Next to the above summarized CAR T cell candidates, several other appealing targets for MM are currently under preclinical investigation. These include CS1 (SLAMF7), which is over-expressed in MM and proven to be effectively targeted by CAR T and CAR NK cells[138–140]. Another candidate is ROR1, which is expressed on MM, among other hematological malignancies. The presumably cancer-specific isoform of CD44, CD44v6, is also being tested preclinically as a CAR target for MM[80].

CD38 as a MM target

In our studies and the topic of this thesis is to target the CD38 molecule, an appealing candidate for MM therapy. CD38 is a type II transmembrane glycoprotein, which functions as an ectoenzyme to catalyzes the conversion of nicotinamide adenine dinucleotide (NAD^+) into nicotinamide (NAD, adenosine diphosphate–ribose (ADPR), and cyclic ADPR[141]. CD38 expression is not specific to MM cells. It can be expressed in immune cells, including a fraction of T cells, NK cells, monocytes, and on granulocytes[120]. It has also been reported to be expressed on lung smooth muscle cells[142], prostate (often luminal)[143] and in purkinje cells of the brain[144]. Nonetheless, for decades, it is known that CD38 has a very high and uniform expression on malignant plasma cells[145] and therefore, despite its somewhat broad expression, this molecule is considered an appealing target for MM treatment[146]. Indeed, the utility of CD38 as a suitable target, as mentioned above, was recently supported by several clinical trials with the CD38-specific human monoclonal antibody daratumumab[119].

In this thesis, we therefore extensively studied the feasibility of generating CD38-CARs and several ways to reduce and control on-target and off-tumor effects of CD38-CAR T cells.

SCOPE OF THIS THESIS

CAR-based immunotherapies are developing rapidly, many suggestions have been made to increase effectiveness and safety in a wider variety of tumor types. Also, efforts are made to develop CAR T cells into a more universal cell product. Future endeavors should elucidate whether CAR T cell treatment enlarges the clinical benefit for multiple myeloma patients.

In this thesis we explored the possibility and efficacy of CAR T cells targeting CD38 in **chapter 2.** To assure safety of CD38 targeting we analyzed CD38-CAR T cell induced cytotoxicity and predict their potential on-target/off-tumor cytotoxicity in a clinically relevant setting.

It has been suggested that the lowering the affinity of CARs could result in selective elimination of cells with high antigen density, while saving other tissues expressing lower levels of the same target. To fine-tune the CD38-CAR, we exchanged the scFvs variable light chain, which resulted in many low affinity CD38-CARs that were extensively tested in **chapter 3**.

Also, the choice of costimulation may affect killing capacity of CAR T cells or increase their overall immunotherapeutic properties. Hence, to better understand the costimulatory requirements of high affinity and attenuated CAR T cells, **in chapter 4** we investigated the role of the intracellular costimulatory domains on the functional properties of CD38-CAR T cells.

In **chapter 5,** we sought for an alternative approach to control the high affinity CD38-CAR T cells. The CAR gene expression was regulated by an inducible (Tet-On) promotor to specifically control CAR expression and its related effectivity.

In the final **chapter 6** we extended our research by studying the feasibility and the potential benefits of introducing CD38-CARs into invariant Natural Killer T (iNKT) cells, which, with their unique antigen recognition pattern, have the benefit to be applied across HLA barriers and to be additionally stimulated through their invariant TCR.

In **chapter 7** the most important results of this thesis are summarized and discussed.

1

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CHAPTER 2

PRECLINICAL EVALUATION OF CD38 CHIMERIC ANTIGEN RECEPTOR ENGINEERED T CELLS FOR THE TREATMENT OF MULTIPLE MYELOMA

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ABSTRACT

Adoptive transfer of Chimeric Antigen Receptor transduced T cells is a promising strategy for cancer immunotherapy. The CD38 molecule, with its high expression on Multiple Myeloma cells, appears a suitable target for antibody therapy. Prompted by this, we used three different CD38 antibody sequences to generate second generation retroviral CD38- Chimeric Antigen Receptor constructs; transduced T cells of healthy donors and Multiple Myeloma patients and evaluated their preclinical efficacy and safety. Irrespective of the donor and antibody sequence, CD38-Chimeric Antigen Receptor transduced T cells proliferated, produced inflammatory cytokines and effectively lysed malignant cell lines and primary malignant cells from acute myeloid leukemia and multi-drug resistant Multiple Myeloma patients in a cell-dose, and CD38-dependent manner, despite becoming CD38 negative during culture. CD38-Chimeric Antigen Receptor transduced T cells also displayed significant anti-tumor effects in a xenotransplant model, in which Multiple Myeloma tumors were grown in a human bone marrow-like microenvironment. CD38-Chimeric Antigen Receptor transduced T cells, also appeared to lyse the $CD38^+$ fractions $CD34^+$ hematopoietic progenitor cells, monocytes, natural killer cells, and to a lesser extent T and B cells but, did not inhibit the outgrowth progenitor cells into various myeloid lineages, and were furthermore effectively controllable with a caspase-9-based suicide gene. These results signify the potential importance of CD38-Chimeric Antigen Receptor transduced T cells as therapeutic tools for $CD38^+$ malignancies and warrant further diminishing their undesired effects using appropriate strategies.

INTRODUCTION

Multiple myeloma (MM), the malignant disorder of antibody producing clonal plasma cells is the second most common hematologic neoplasia worldwide[1]. Despite four decades of drug innovation, MM remains incurable with chemotherapy. Furthermore, the prognosis of MM patients, who become refractory to recently developed novel agents, is very poor[2]. On the other hand, clinical and experimental data collected over the past decades suggest the possibility to successfully treat MM through (cellular) immunotherapy[3,4]. The curative potential of cellular immunotherapy in MM is illustrated by the induction of long-term sustained remissions after allogeneic stem cell transplantation (SCT) or donor lymphocyte infusions (DLI) in a subset of patients[5,6]. A highly appealing and more specific immunotherapy strategy for cancer is the adoptive transfer of cytotoxic T cells (CTLs) that are genetically engineered to express Chimeric Antigen Receptors (CAR)[7,8]. A CAR is an artificial hybrid receptor, in which the antigen recognizing domain of a tumor-reactive monoclonal antibody is fused with T-cell signaling domains. Upon retro- or lentiviral transduction of CTLs, CARs expressed on the cell surface redirect the CTLs toward the original target of the antibody in a non-HLA restricted manner[7,8], providing the possibility to apply the therapy regardless of the HLA type of the patient. Currently the most successful CAR-approaches are based on targeting the CD19 molecule, which is broadly expressed in several B cell malignancies but not on malignant plasma cells of MM patients. Among a few potential CAR candidates for MM[9], the CD38 molecule, with its high and uniform expression on malignant plasma cells, has long been suggested a suitable target for antibody therapy of MM. The utility of CD38 as a suitable target has indeed been supported by the results of recently initiated clinical trials in which MM patients were safely and effectively treated with the CD38-specific human monoclonal antibody daratumumab[10].

Encouraged by these clinical results, we now started to explore the feasibility of development of a CAR T cell therapy based on targeting the CD38 molecule. Using variable heavy and light chain sequences of three different human CD38 antibodies, we generated three different CD38-CARs. We transduced T cells from healthy individuals and MM patients with CD38-CARs and evaluated them for essential functions such as antigen-specific proliferation and cytokine production, for *in vitro* and *in vivo* anti-tumor efficacy and for potential undesired effects such as targeting normal $CD38^+$ cell fractions in the peripheral blood and bone marrow. We also evaluated the feasibility of controlling CD38-CAR T cells by introduction of a caspase-9 based suicide gene.

METHODS

Retroviral constructs

CD38-CAR Constructs The sequences of the variable light and heavy chain of three different human CD38 antibodies, which are distinct than the recently documented daratumumab[10] were kindly provided by Genmab. The sequences were synthetically produced (GeneArt Invitrogen) in a scFv (single chain variable fragment) format with a signaling sequence and

a G_4S linker. The CAR design is depicted in figure 1A. A G_4S linker separates the heavy and light chain sequences. The CD8a leader sequence placed in front of the scFv sequence facilitates signaling to the plasma membrane. To complete the CAR construct, the scFv was cloned in frame with a CD8a transmembrane domain, a 4-1BB and a CD3ζ sequence, identical to the second generation CAR design that has been used by Porter *et al*.[11]. Downstream of the CAR, an IRES induces the simultaneous transcription of the neomycine resistance gene. The generated CAR constructs were cloned into a pBullet retroviral vector under a CMV promoter and sequence was confirmed with Big dye v3.1 terminator and ABI sequencer (Biosystems). *Caspase-9 based suicide gene construct.* The inducible caspase-9 retroviral pMSCV plasmid was kindly provided by David Spencer (Addgene, plasmid 15567)[12,13].

Generation of retroviral particles

The CAR constructs, gag-pol (pHIT60), and envelop (pCOLT-GALV) vectors (Roche) were transfected using Fugene-6 transfection reagent (Roche) into Phoenix Ampho packaging cells, cultured in DMEM + 10%FCS + antibiotics (penicillin;100 U/ml, streptomycin; 100µg/ml). Three days after transfection, cell free supernatants containing retroviral particles were collected. The virus particles were concentrated using the RetroX kit following the instructions of the manufacturer (ClonTech) and were frozen at -80°C until use.

Retroviral CAR Transduction into T cells

PBMCs from healthy donors or MM patients were stimulated with αCD3/CD28 Dynabeads™ (1×10^6 beads/10^6 cells) (Invitrogen) in RPMI-1640 + 10% FBS. After 48 hours, a total of 1×10^6 cells were retrovirally transduced by incubation of 50 µl of concentrated CAR retrovirus per 1×10^6 cells and 8 µg/ml Polybrene. After immediate centrifugation for 30 minutes at 32°C, the cells were cultured at 37°C and received a second hit after 24 hours. To obtain a pure population of CD38-CAR$^+$ T cells, transduced T cells were selected and expanded using culture medium (RPMI-1640, 10% human serum, antibiotics) supplemented with 800 µg/ml neomycin (Gibco, Karlsruhe, Germany) and 50 IU/ml rhIL-2 (Proleukin®, Novartis). After one week a fraction of expanding CAR-transduced T cells were sorted for CD4$^+$ and CD8$^+$ cells using MACS (Miltenyi Biotec). Unsorted and sorted T cells were expanded in culture medium containing IL-2 (50 IU/ml) and were restimulated for several rounds (14 days/round) using a feeder cell/cytokine mixture consisting of irradiated EBV cell lines (50 Gy) and allogeneic PBMCs (25 Gy),100 U/ml IL-2 and 1 ng/ml Phytohemagglutinin-L (PHA-L) (Sigma).

Retroviral transduction of CAR positive T cells with inducible caspase-9

Selected and expanded CAR-positive T cells were transduced with pMSCV-F-del Casp9. IRES.GFP, after stimulation with αCD3/CD28 Dynabeads™ (1×10^6 beads/10^6 cells) as described above. GFP-positive transduced cells were sorted with FACS-Aria II sorter and

were subsequently treated with increasing concentrations of B/B homodimerizer (Clontech, identical to AP20187 ARIAD Pharmaceuticals, Cambridge, MA)[12] to test the functional expression of the inducible caspase-9. After dimerization cells were analyzed for their GFP, AnnexinV and Topro3 expression with flow cytometry.

Cell lines

Unmodified or luciferase (Luc-GFP)-transduced human MM cell lines, U266, UM9, LME-1, UM9, MM1.S, U266, L363 and UM3, human leukemic cell lines HEL, MOLM13, CEM and Daudi were cultured in RPMI-1640 (Invitrogen) + 10% FBS (Integro BV) + antibiotics (penicillin;100 U/ml, streptomycin; 100 µg/ml) as described[14]. HUVECs were cultured in EGM2 medium (Lonza). All cell lines were authenticated by surface expression of appropriate receptors maximal six months before the most recent experiment.

Bone marrow Mononuclear cells (BM-MNC) from MM and AML patients

Bone marrow mononuclear cells containing 5-20% malignant plasma cells or ~50% AML blasts were isolated from bone marrow aspirates of MM/AML patients through Ficoll-Paque density centrifugation and cryopreserved in liquid nitrogen until use. All bone marrow and blood sampling from the patients was performed after informed consent and approved by the institutional medical ethical committee.

PBMC from healthy individuals

PBMCs were isolated from Buffy coats of healthy blood-bank donors by Ficoll-Paque density centrifugation after informed consent and approval by the institutional medical ethical committee.

Flow cytometry

Flow cytometry assays were performed on FACS Calibur, CantoII or LSR Fortessa (BD). Different cell subsets in PBMC or in BM-MNC in various flow cytometry-based assays were determined using fluorescein conjugated antibodies specific for human CD3, CD4, CD7 CD8, CD13, CD14, CD16, CD19, CD33, CD38, CD45, CD56 and CD133, CD138 (BD Bioscience). CAR surface expression by flow cytometry was done with Biotinylated protein L (GeneScript; Piscataway, NJ) and phycoerythrin (PE)-conjugated streptavidin (BD Bioscience) as described[15].

Flow cytometry-based cell lysis assays

To detect the lysis of various cell subsets by CART cells in whole BMNC or in PBMC, serial dilutions of CART cells were incubated with CFSE labeled BMMNC or PBMC for 24 hours. The cells were then harvested, stained for different CD markers and topro3 or LIVE/DEAD® Fixable Near-IR (Life Technologies L10119) and were quantitatively analyzed through volume-equalized measurements using a FACS Canto flow cytometer. For each

cell subset identified with a CD marker, $CFSE^+$, $viable^+/Topro3^-$ cells were counted as surviving target cells. Percentage cell lysis in a treated sample was calculated as follows and only if the analyzed target cell population contained >500 viable cells in the untreated samples. % lysis cells = 1 − (absolute number of surviving cells in treated wells / absolute number of surviving cells in untreated wells) × 100%.

BLI-based cell lysis assays

To determine the lysis of Luc-GFP-transduced human malignant cell lines by CD38-CART cells, serial dilutions of mock or CD38-CART cells were co-incubated with the malignant cell lines. The luciferase signal produced by surviving malignant cells was determined after 16-24 hours with a luminometer SpectraMax, Molecular Devices within 15 minutes after the addition of 125 µg/mL beetle luciferin (Promega)[14]. The percent lysis was then calculated as in flow-based cytotoxicity assay above.

Tritium-based proliferations assays

To determine (CD38-dependent) proliferative capacity, Mock and CAR T cells were seeded in triplicates in 96-well plates in 3:1 ratio with 50 Gy irradiated UM9 cells. [^{3}H]Thymidine (^{3}H -TdR) (1 µCi per well; Amersham Biosciences) was added after 72 h and the cells were cultured for another 16 h before harvesting. ^{3}H-TdR incorporation was determined by liquid scintillation[16].

Cytokine measurements

To determine the broad array of cytokines produced by CAR T cells, we used the Cytokine Bead Array (CBA) Human Th1/Th2 cytokine kit (BD) according to manufacturer protocol. In brief, a mixture of capture beads (IL-2, IL-4, IL-5, IL-10, TNF and IFN-γ), PE-detection reagent and cell supernatant were incubated for 3 hours. Beads were washed and analyzed by a standardized flow cytometry assay.

Transmigration assay

To analyze the migration capacity of Mock and CAR T cells, the Cytoselect Leukocyte Transmigration Assay (Cell Biolabs, Inc) was used according to manufacturer's protocol. In brief, HUVECs were seeded on membranes in a 24 well plate, after 48 hours Mock and CAR T cells were seeded and migration over the membrane was detected with leukocyte tracker labeling.

Hematopoietic precursor cell (HPC) growth inhibition assays

HPC growth inhibition assays were performed as described previously[17]. Briefly, either 10, 100 or 1000 $CD34^+$ sorted BM cells were mixed with effector T cells at different T:BM cell ratios in 0.2 mL of RPMI culture medium. The cells cultured for 4 hours to create cell contact and then resuspended to a final volume of 1.2 mL with semisolid Methocult (Stem

cell technologies, H4534). One milliliter of the semisolid suspension was then plated in 30-mm plastic dishes and incubated at 37°C in 5% CO_2. After 21 days, the number of colony-forming unit-granulocytes (CFU-G), and CFU-monocytes (CFU-M), were scored under a microscope.

Experimental animals

RAG2$^{-/-}\gamma c^{-/-}$ mice used in this study were originally obtained from the Amsterdam Medical Center (AMC, Amsterdam, the Netherlands). The mice were bred and maintained in filter top cages under specified pathogen-free conditions at the Central Animal Facility (GDL, Utrecht University, Utrecht, the Netherlands) and received sterile water and radiation-sterilized food pellets ad libitum.

In vivo efficacy of CD38-CAR T cells against MM tumors growing in a humanized microenvironment.

To create a human bone marrow-like environment in mice, hybrid scaffolds consisting of three 2- to 3-mm^3 biphasic calcium phosphate particles were coated *in vitro* with human mesenchymal stromal cells (MSC)(2×10^5 cells/scaffold). After a week of *in vitro* culture in a bone-formation promoting medium, humanized scaffolds were seeded with CD38$^+$ UM9 cells (1×10^6 cells/scaffold) to allow adherence to the hu-scaffolds and after 16 hours implanted subcutaneously into the mice, as described previously[14,18]. Seven days after implantation, when the tumors became detectable in the scaffolds by BLI, different groups of mice received tail i.v. injections of CD38-CAR T cells or with mock-transduced CAR T cells (20×10^6 cells/mice). The injections were repeated on day 9 and 13. Tumor growth was monitored by weekly BLI measurements as described previously[18].

All animal experiments were conducted after acquiring permission from the local ethical committee for animal experimentation and were in compliance with the Dutch Animal Experimentation Act.

Immunohistochemistry

Tumor were dissected from mice and fixed in formalin, subsequently decalcified with Cal-Rite™ (Thermo Scientific) and paraffin-embedded. Retrieved sections were blocked with 0.3% H_2O_2 in methanol. Sections were boiled for 10 minutes in a citrate buffer, pH 6, and then blocked with serum-free protein block. The slides were incubated overnight at 4°C with anti-38 (Monosan BLD2) or CD138 (IQP-153P). The sections were counterstained with hematoxylin, washed, and subsequently dehydrated through graded alcohol, cleared in xylene, and coverslipped.

RESULTS

Generation of CD38-CART cells

We used the variable heavy and light chain sequences of three different CD38 antibodies with CD38 binding affinities comparable to daratumumab (Table S1), which is now being tested in clinical trials. T cells from healthy PBMC were transduced with the different CD38-CAR genes or with the empty vector (mock) separately. After selection of transduced cells to high purity by neomycin treatment, the surface expression of CARs was determined by incubating the T cells with biotinylated bacterial protein L, which specifically binds to the variable region of kappa light chains of antibodies[15]. Indirect staining with PE-conjugated streptavidin revealed the expression of all three CARs on >95% of the T cells, whereas T cells transduced with an empty vector (mock-transduced T cells) displayed only background staining (Fig. 1B left panel). The CAR-transduced cells contained variable levels of both $CD4^+$ and $CD8^+$ cells (Fig. 1B; right panel).

CD38-dependent proliferation and cytokine secretion of CD38-CART cells

To analyze the proliferative and functional properties, the neomycin-selected, highly purified CD38-CART cells were expanded using irradiated feeder cells in the presence of PHA-L and IL-2. While the mock T cells initially expanded better than the CD38-CART cells (Fig. 1C; left panel), the growth disadvantage of CD38-CART cells disappeared in the second expansion round (Fig. 1C; right panel), indicating that transduction of CD38-CAR construct did not affect the proliferative capacity of T cells. We then tested whether CD38-CART cells can be activated by CD38-triggering. To this end, we co-cultured mock- and CD38-CAR-transduced T cells with irradiated $CD38^+$ MM cell line UM9 and used the $CD38^-$ MM cell line U266 as a control (Fig. 1D; left panel). CD38-CART cells, but not mock T cells, specifically proliferated and produced IFN-γ, TNF-α and IL-2 (Fig.1D; right panel), but not IL-4, -5 and -10 (data not shown) upon stimulation with UM9 cells. These results indicated that CD38-CART cells had no defects in cytokine production but displayed a typical Th1-like cytokine response upon target recognition. Furthermore, the $CD38^-$ cell line U266 was unable to stimulate CD38-CART cells, demonstrating the proper antigen-specific function of CD38-CART cells.

CD38-dependent lysis of MM cell lines by CD38-CAR T cells

To determine the CD38-dependent lysis of malignant cells by CD38-CART cells, we first used luciferase-transduced MM cell lines with variable CD38 expression levels in BLI-based cytotoxicity assays[14,19]. As expected, there was no CD38-CAR-specific lysis of the $CD38^-$ U266 cell line (Fig. 2A). In contrast, all three CD38-CART cells, but not mock-T cells, effectively lysed $CD38^+$ MM cell line UM9 in a cell-dose dependent manner (Fig. 2B), showing the feasibility of generating effective CART cells with any of the CD38 antibody

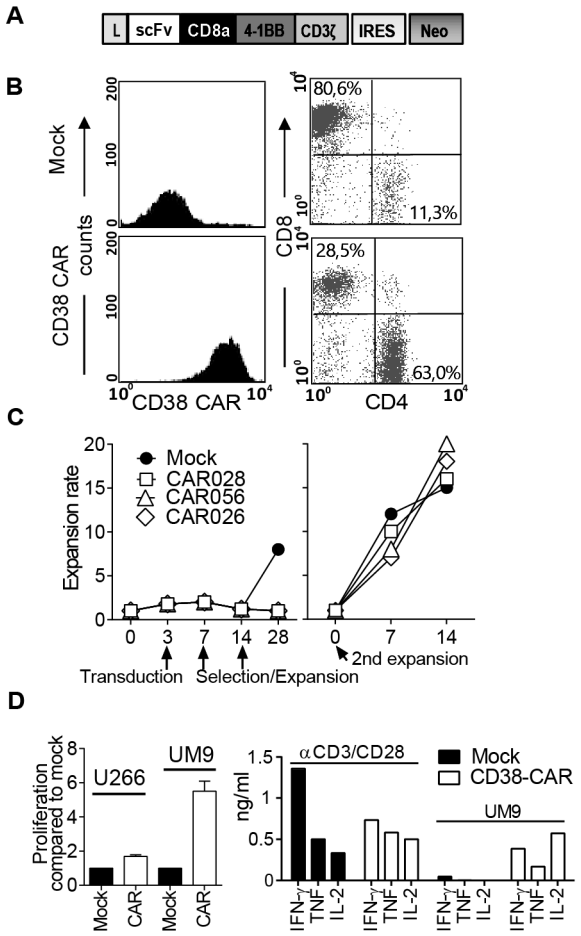

Figure 1. CD38-CAR construct and CD38-CART cell phenotype. (A) Schematic overview of the CD38-CAR construct. CD38-scFv sequence is based on three different antibody sequences (028, 056 and 26, see also supplementary Table S1), with CD8a as transmembrane domain and 4-1BB and CD3ζ as intracellular domains. (B) CAR expression on the cell surface of healthy donor T cells was determined by binding of biotinylated protein L to the scFv domain (left panel), stained with PE labeled streptavidin. Depicted are the results for CD38-CART cells generated with CAR056, representative for all three CARs. The expression of surface markers CD4 and CD8 on (right panel) was determined by fluorescence-labeled monoclonal antibodies. (C) The expansion of mock and CD38-CART cells after transduction (left panel) and after the second round of stimulation (right panel; new stimulation set at "0"). (D) The relative ^{3}H-thymidine uptake (left panel) of mock and CD38-CART cells after 72 h stimulation with the CD38^{+} MM cell line UM9: responder ratio of 3:1. Error bars represent mean + SEM, n=3. The results are expressed as relative stimulation index, compared to mock and considered significant if the stimulation index is >=3. The cytokine secretion (right panel) from of mock and CD38-CART cells stimulated with αCD3/CD28 beads or CD38-specific with the MM cell line UM9. The cytokine secretion was measured with the flow cytometry-based CBA kit (BD) in the cell free supernatants after 24 hours of stimulation. Graph shows the secretion of IFN- γ, TNF and IL-2. Secretion of IL-4, -5 and -10 were below the detection limits. These data are therefore not shown in this figure. Similar results were obtained in two independent assays.

sequences we used. Since there was no functional difference between the three different CD38-CARs (028, 056, 026), we continued our investigation with one type of CD38-CART cell (CAR056). Flow cytometry and BLI-based cytotoxicity assays, executed using other malignant cell lines expressing various levels of CD38 (Supplementary Fig. S1) as target cells revealed a good correlation between the CD38 expression and CD38-CART cell-mediated lysis (Fig. 2C). Though one AML cell line, the Burkitt's lymphoma-derived cell line Daudi as well as normal T cells appeared less sensitive to CD38-CART cell mediated lysis as compared to MM cell lines with similar levels of CD38 expression (Fig. 2C).

Lysis of primary MM and AML cells by CD38-CART cells.

To test the efficacy of CD38-CART cells against primary MM and AML cells, we used a previously described flow cytometry-based *ex vivo* cytotoxicity assay, in which the lysis of malignant cells is tested, directly in the BM-MNCs without isolating them from other cells.[20] As depicted in Figure 3A, primary $CD138^+CD38^+$ MM cells of three different MM patients, who were refractory to treatment with lenalidomide, and bortezomib (left panel), were effectively lysed by CD38-CART cells, but not by mock-transduced T cells. Similarly in the BM-MNCs of two Acute Myeloid Leukemia (AML) patients malignant cells, which were identified as $CD13^+$ $CD45^+$ cells and expressed either low/intermediate (patient 1) or high CD38 (patient 2) were effectively lysed by CD38-CART cells (Fig 3A). Finally, CD38-CART cells that were generated (Fig 3B) from a MM patient were effective towards autologous malignant MM cells in BM-MNCs indicating the feasibility of generating effective CD38-CART cells also from MM patients.

Fully-functional CD38-CART cells are negative for CD38

While CD38-CART cells had no apparent functional deficiencies, a phenotyping assay revealed that they, despite a mixed effector/central memory phenotype, lost the expression of CD38 (Fig. 4A). Interestingly, when we co-cultured CD38-CART cells with an autologous CD19-CART cell population, these CD19-CART cells also became largely negative for CD38 expression but fully maintained their capacities to proliferate, secrete cytokines and kill the relevant target cells in a CD19 dependent fashion (supplementary Figure S2), indicating that the loss of CD38 was not associated with detectable T cell dysfunction. Nonetheless, since CD38 molecule could also play a role in migration, we also evaluated whether CD38 negative CD38-CART cells would properly migrate through endothelial layers in a transwell migration assay (fig 4B). These assays revealed no differences between the mock-transduced, CD38-positive and CD38-CAR-transduced CD38-negative T cells, ruling out an apparent migratory dysfunction of CD38-CART cells.

In vivo efficacy of CD38-CART cells against MM tumors growing in a humanized microenvironment

To substantiate the *in vitro* results, we questioned whether the CD38 negative CART cells could mediate *in vivo* anti-MM effects after systemic injection in our recently developed

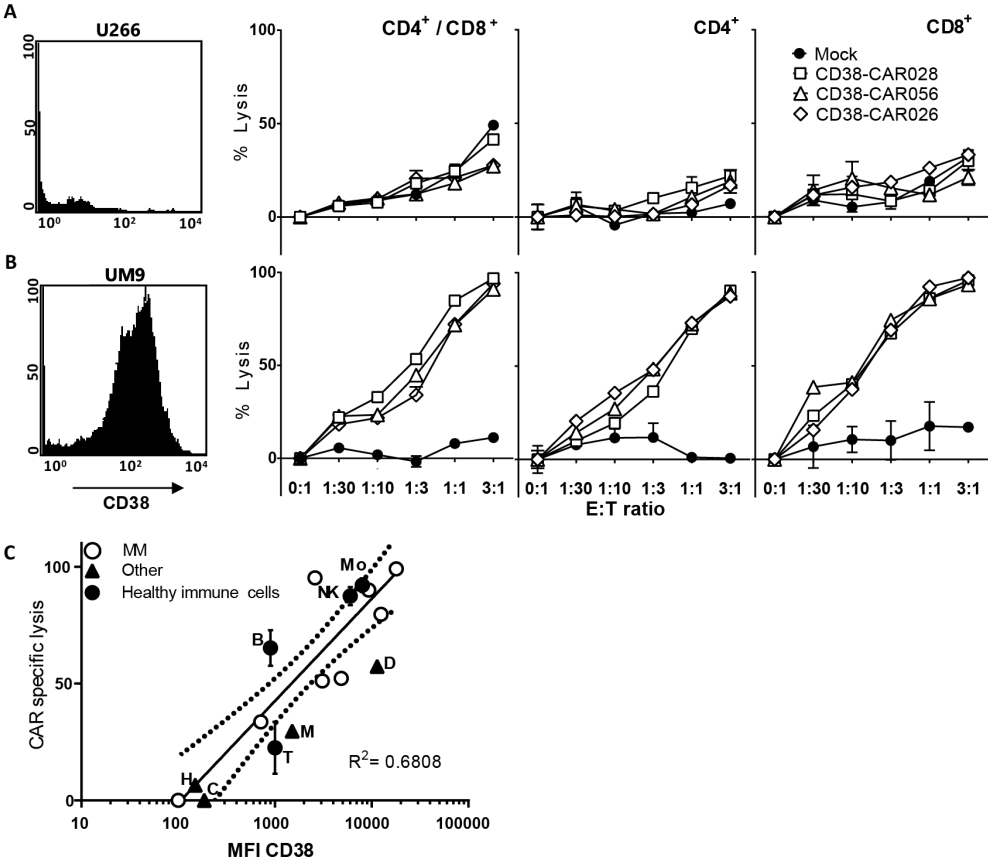

Figure 2. Efficacy of CD38-CART cells to lyse MM cell lines. In 24 h cytotoxicity assays, three different CD38-CART cells were tested against two MM cell lines with different CD38 expression levels (A) U266, a CD38-negative cell line, (B) UM9, a CD38+ cell line. Effector:Target ratios are indicated. Target cells per well were 10,000 MM cells. Closed circles (●) indicate mock and open squares, triangle and diamond (□,◇,△) indicate CAR028, 056 and 026. Error bars indicate mean + SD. (C) Correlation between mean fluorescent intensity (MFI) of CD38 on target cells and consequential CD38-CAR specific lysis. CD38-CART cells (CAR056) were co-cultured with leukemic cell lines and allogeneic healthy donor PBMCs. The resulting lysis in a 3:1 ratio was determined with BLI or flow cytometry, minus the spontaneous lysis caused by mock T cells. Open circles (o) indicate MM cell lines (LME-1, UM9, MM1.S, U266, L363 and UM3), triangles (▲) indicate AML (HEL, MOLM13), T lymphoblast (CEM) and Burkitt's Lymphoma (Daudi), closed circles (●) indicate healthy immune cells (T= T cells, B=B cells, NK= NK cells, Mo= monocytes, C=CEM, H=HEL, M=MOLM13, D=Daudi), Error bars represent mean + SEM of duplicate measurements.

model in Rag2$^{-/-}\gamma c^{-/-}$ mice, in which a humanized BM like-niche for MM cells are generated by s.c. implantation of ceramic scaffolds coated with human bone marrow stromal cells (huBMSCs)[14,18] (Fig4). Thus, we implanted huBMSC-coated scaffolds seeded with luciferase-transduced UM9 MM cells in the back of the mice (6 scaffolds per mouse). Upon detection of luciferase signal by BLI, we treated the mice by i.v. injections of CD38-CART cells using a previously established treatment scheme.[21] Mock-transduced T cells were used as

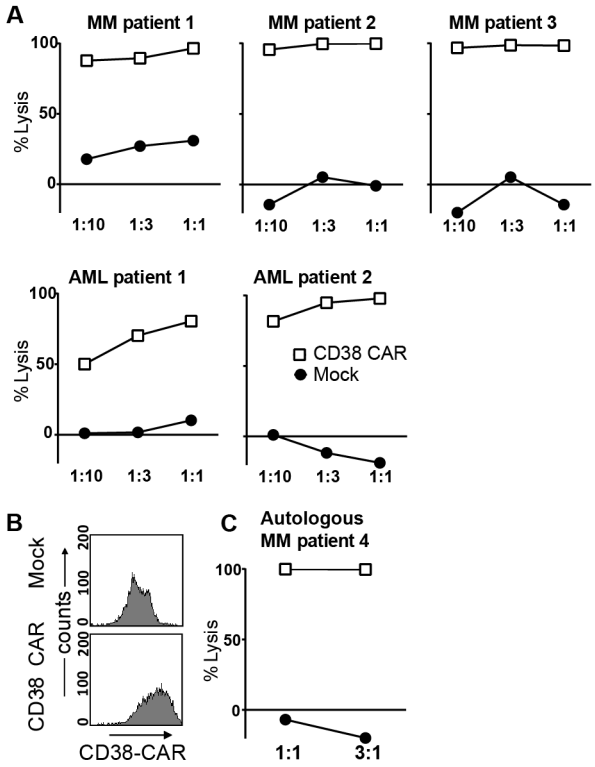

Figure 3. Efficacy of CD38-CART cells generated from healthy individuals to lyse primary MM cells. (A) Bone marrow derived mononuclear cells (BM-MNCs) of three MM patients, all three refractory to lenalidomide, and bortezomib and BM-MNCs of two AML patients were co-incubated with no, mock- or CD38-CART cells generated from healthy PBMC for 16 h. Closed circles (●) indicate mock and open squares (□) indicate CAR056T cells (representative for all CARs). The graphs depict the resulting lysis of $CD138^+/CD38^+$ cells (MM) or $CD13^+/CD7^+/CD45dim/CD38^+$ (AML1, moderate CD38 expression) and $CD33^+/CD133^+/CD45dim/CD38^+$ (AML2, high CD38 expression) in three E:T ratios. The % lysis in these flow cytometry assays was calculated as described in the methods section. (B) Efficacy of CD38-CART cells generated from a MM patient: CAR expression on the cell surface of patient's T cells was determined by flow cytometry with protein L staining (see also figure 1). (C) Bone marrow derived mononuclear cells (BM-MNCs) of the MM patient were co-incubated with autologous mock- or CD38-CART cells for 16 h. The graph depicts resulting lysis of $CD138^+/CD38^+$ cells in two ratios, determined in flow cytometry based assays.

controls. As illustrated in Figure 4B, in the control group treated with mock T cells, tumors showed a fast progression. Although not curative, treatment of the tumor-bearing mice with CD38-CART cells induced a significant anti-tumor effect (Fig. 4B,C) underscoring the potential of CD38-CART cells to properly infiltrate and lyse MM tumors growing in their natural, protective niche. Post mortem analyses revealed that the remaining $CD138^+$ tumors were still positive for CD38 (Fig. 4D), thus ruling out tumor escape due to "antigen loss" variants.

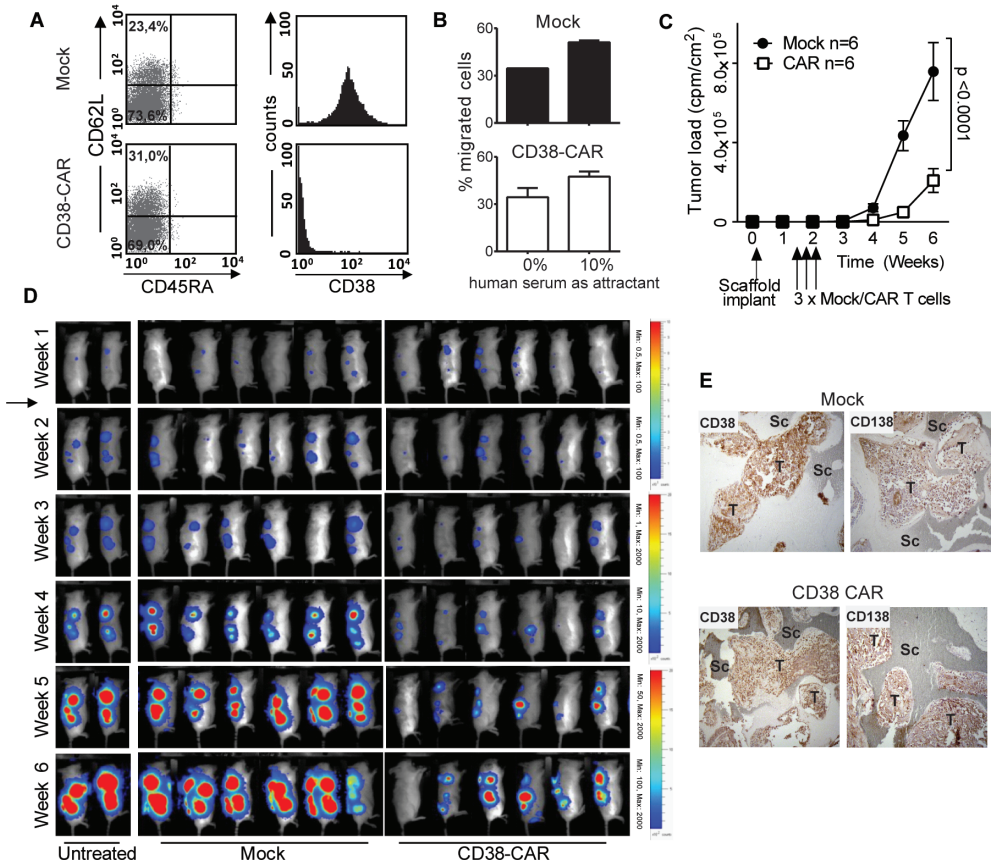

Figure 4. Tumor growth in Mock- and CD38-CART cell treated mice. (A) Analysis of CD38-CART cells after two weeks of *in vitro* culture, with fluorescence labeled monoclonal antibodies for CD45RA and CD62L and CD38. (B) Leukocyte transmigration assay, where mock and CART cells were cultured in a transwell system in the inserts with endothelial (HUVEC) cells, which were activated with TNF-α. Spontaneous TNFα-induced transmigration was compared to active migration induced by 10% of human serum in the lower compartment. % migrated cells = (Relative Fluorescence Units (RFU) of cells in lower compartment / RFU of total cells in both compartments) * 100%. (C) Analysis of tumor load in mice by quantification of BLI measurements. Each group contained six mice, each harboring 6 scaffolds. Results are mean tumor load (cpm/cm^2) of 6 mice per group. Closed circles (●) indicate Mock and open squares (□) indicate CAR056. The error bars represent mean + SEM, n=6. The differences between groups were analyzed after week 6 in, an unpaired student's T test, $p < 0.0001$ (D) Bioluminescent imaging of mice on right side, mice were implanted with fully humanized BMSC scaffolds each coated with 1×10^6 UM9-GFP-Luc tumor cells. 7, 9 and 13 days after implantation, mice were i.v. injected with 20×10^6 Mock or CD38-CART cells. (E) Representative immunohistochemistry figure, remaining tumor were stained with CD38 and CD138 antibody, T = tumor, sc = scaffold.

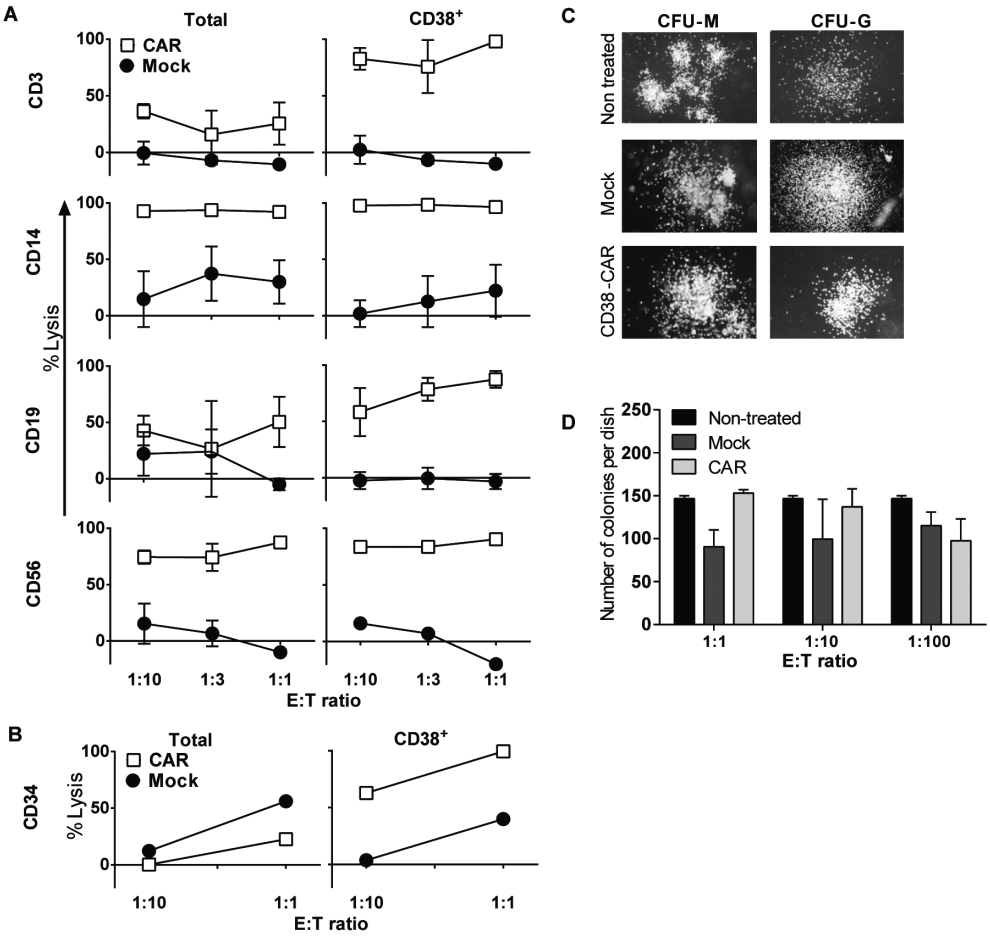

Figure 5. The impact of CD38-CART cells on non-malignant hematopoietic cells in BM and outgrowth of Hematopoietic cell lineages. (A) BM-MNCs of 3 MM patients were co-incubated with none, mock- or CD38-CART cells for 16 h. The graphs depict the resulting lysis of the total or the $CD38^+$ fractions of $CD3^+$ (T cells), $CD56^+$, (mainly NK cells), $CD14^+$ (monocytes) and $CD19^+$ (B cells) cell subsets in three ratios, determined with flow cytometry and calculated as described in the methods section. Results are 3 individual experiments combined. Closed circles (●) indicate Mock and open squares (□) indicate CAR056. Error bars represent mean + SEM, n=3. (B) $CD34^+$ fraction of BM-MNCs from healthy donors were co-incubated with none, mock- or CD38-CART cells for 4 h at different T:BM cell ratios before transferring into the semisolid HPC culture medium. After the incubation, cells were analyzed by flow cytometry for surviving $CD34^+$ cells with CD38 expression. The graphs depict the resulting lysis of the total or the $CD38^+$ fraction of $CD34^+$ cells. Closed circles (●) indicate Mock and open squares (□) indicate CAR056. (C) After 14 days of culture in plastic dishes, colony-forming unit-monocytes (CFU-M), and CFU-granulocytes (CFU-G) were visible, (D) the number of CFU-M, and CFU-G colonies were determined microscopically. Results of a representative experiment are shown mean + SD.

Impact of CD38-CART cells on CD38$^+$ normal hematopoietic cells and hematopoietic progenitor cells.

Besides the high expression levels in MM cells, the CD38 molecule is expressed at intermediate levels on a subset of hematopoietic progenitor cells[22] and on a fraction of normal hematopoietic cells including activated T cells, NK cells, B cells and monocytes. We therefore evaluated the possible negative impact of CAR-T cells on these cell subsets by co-incubating unsorted BM-MNCs with CD38-CART cells. CD38-CART cells appeared to eliminate the CD38$^+$ fractions of mature T, B, NK and monocyte cell subsets (Fig. 5A) and the CD38$^+$ fraction of CD34$^+$ cells (Fig. 5B) in a 4 hour assay. The lysis of CD34$^+$CD38$^+$ cells had however no influence on the development of colony-forming unit-monocytes (CFU-M), and CFU-granulocytes (CFU-G) in a 14-day hematopoietic precursor cell (HPC) colony forming assay[23,24] (Fig. 5C and D).

Specific elimination of CD38-CART cells using a suicide gene (iCasp9)

Although CD38-CART cells did not lyse the CD38 negative fractions of mature hematopoietic cells and did not inhibit the outgrowth of these cell populations, still a cautious approach toward the clinical application is required. Therefore, as a first step towards a more safe application of CD38-CART cells, we tested the possibility to control them with a suicide gene based on the inducible caspase-9 (iCasp9) gene that is activated with a small dimerizer molecule AP20187 (B/B)[12]. Thus, we inserted an iCasp9 vector containing a GFP marker gene into the CD38-CART cells by retroviral transduction. Around 50% of the CD38-CART cells were transduced as detected by GFP expression (figure 6A, upper panel) When tested without sorting the iCasp9 transduced (GFP+) cells, all iCasp9 transduced, GFP+ but none of the iCasp9 non-transduced, GFP-CD38-CART cells were eliminated upon incubation with the dimerizer AP20187 (Fig. 6A lower panel). As expected, the dimerizer treatment also resulted in a proportional decrease in the lysis of the MM cell line UM9. (Fig. 6B). There was still a remaining lysis due to the surviving iCasp9 negative CD38-CART cells, indicating that the triggering of suicide gene induced no bystander damage to the cells in the close vicinity. When tested after sorting of GFP+ cells (Fig 6C and 6D), almost all GFP+ cells died after treatment with the dimerizer (Fig. 6C) and there was no CD38 specific lysis left (Fig 6D), confirming the results obtained by previous studies[12,25], and suggesting the possibility to control CD38-CART cells using the iCasp9 suicide gene without undesired consequences.

DISCUSSION

While cellular immunotherapy of hematological malignancies has been applied for many decades in the most non-specific form as allo-SCT or DLI, it has recently entered a more specific level of innovation with several encouraging strategies, including vaccination with antigen-loaded dendritic cells or adoptive immunotherapy with TCR-gene transferred T

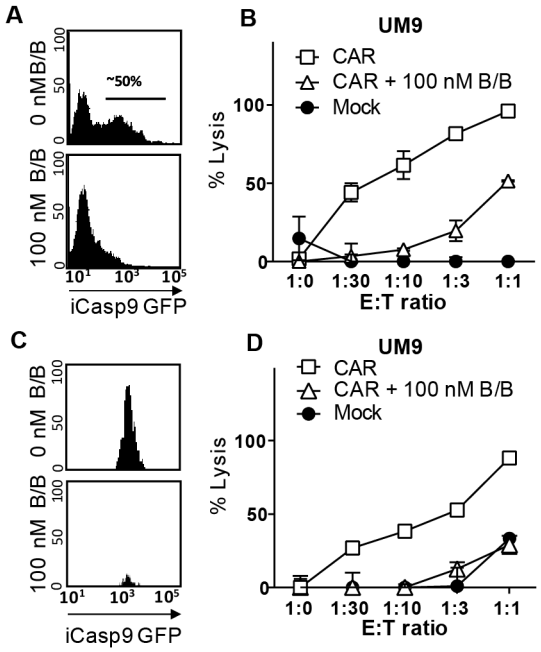

Figure 6. Dimerizer AP20187-mediated elimination of the iCasp9 suicide gene transduced CD38-CAR T cells. (A) Flow cytometry histogram plots, CD38-CAR T cells that were additionally transduced with the iCasp9-GFP construct. Upper panel shows the untreated cells 50% GFP$^+$; lower panel the cells treated with 100nM dimerizer AP20187 (B/B). (B) The lysis of UM9 cell line by iCasp9-transduced CD38-CAR T cells that were untreated or treated with the dimerizer. The significant reduction of GFP$^+$ cells (A) is a consequence of cell death activated by the dimerizer B/B. Note (in B) the decrease in cytolysis is proportional to the elimination of the suicide gen transduced cells (50% of all CAR-positive cells in A. The residual cytolysis is thus caused by the CAR-positive cells that were not transduced with iCasp9 n=2, mean + SD. (C) CD38-CART iCasp9-GFP-high sorted cells. Upper panel shows the untreated cells 100% GFP$^+$; lower panel the cells treated with 100nM dimerizer B/B. (D) The lysis of UM9 cell line by iCasp9-High-CD38-CART cells that were untreated or treated with the dimerizer. Closed circles (●) indicate Mock and open diamond (◇) and triangle (△) indicate CAR056 – and + B/B. n=2, mean + SD.

cells, tumor infiltrating T cells and more recently with CTLs endowed with tumor-reactive CARs. Among these strategies, CAR-based therapies are perhaps the most appealing, as CART cells recognize their target antigens in an MHC-independent manner. Set out to develop a CAR-based strategy for MM, we have been encouraged by the highly promising clinical results of the therapy with daratumumab, which targets CD38, a type II transmembrane glycoprotein, expressed with high and uniform levels in most, if not all, MM cells in all stages of the disease[10]. Recently daratumumab has been applied to several patients at moderate to high doses and for prolonged periods with little or no toxicity. This, despite the fact that CD38 molecule is also expressed, albeit at lower levels, on a fraction of hematopoietic cells, cerebellar purkinje cells, liver and lung smooth muscle cells, and insulin-secreting β cells of pancreas[22]. Our study was therefore designed to

test the feasibility, potential efficacy and pitfalls of a CD38-based CART cell approach for MM. To investigate the feasibility of generating CD38-CARs, we started the investigation using three distinct human CD38 antibodies, which displayed similar binding affinities to CD38 as daratumumab (Supplementary Table S1). Based on successful usage of 4-1BB-containing CARs in recent studies[26–28] we have constructed CARs containing 4-1BB (CD137) co-stimulatory and CD3ζ activating domains. Our results demonstrate the successful generation of CD38-CARs and CD38-CART cells regardless of the antibody sequences. T cells transduced with these CD38-CARs are highly proliferative, produce inflammatory Th1 like cytokines and, most importantly, are effective in killing malignant cells and normal hematopoietic cells in a CD38-dependent fashion, with some subtle differences between cell lines or hematopoietic cell types.

More importantly, CD38-CART cells appeared capable of eliminating primary $CD38^+$ MM cells of patients who became resistant to various chemotherapies. This suggests that CD38-CAR therapy could be a viable option for patients with little or no further chemotherapy options. These *in vitro* data were substantiated by the results obtained in our *in vivo* model. Although we did not observe the complete eradication of MM cells in our *in vivo* assays, we need to note that, since our CD38-CART cells appeared to lose their CD38 expression upon culture, we primarily designed our *in vivo* assays to determine the anti-tumor efficacy of these CD38 negative, but long-term cultured CD38-CART cells. This may have negatively influenced the anti-tumor efficacy, since it is known that long-term cultured T cells rapidly lose their *in vivo* persistence capacities[29,30]. In addition, and perhaps even more important, in our model, unlike all previously reported CAR studies, the human MM tumors are grown to larger masses in a fully humanized BM microenvironment. The MM microenvironment is known to provide essential signals for survival, growth and more importantly immune resistance of MM cells.[14,18,31,32] Since our model includes some of the microenvironment related aspects, our results suggest that it the efficacy of CART cell treatment could be improved if the therapy would be combined with immune checkpoint inhibitors and or with survivin and/or MCL-1 inhibitors which are effective modifiers of cell adhesion mediated immune resistance (CAM-IR) induced by tumor microenvironment[14].

Unlike a number of earlier reports, which mainly focused on the anti-tumor efficacy of CD38-CART cells,[33–35] we devoted a considerable part of our investigation on identifying the potential drawbacks and risks of the CD38-CART cell therapy. Although CD38-CART cells eliminated the $CD38^+$ fractions of immune cell subsets as well as the $CD38^+$ fraction of hematopoietic progenitor cells, we observed no inhibition of the outgrowth of hematopoietic lineages from $CD34^+CD38^-$ progenitor cells. Furthermore, CD38-CART cells did not induce complete depletion of mature hematopoietic cells in the periphery. The CD38 negative fractions of important immune cells, such as B and T cells, were unaffected as well. These results suggest that the therapy will spare sufficient numbers of T and B cells to maintain their functions. However, since CD38 is a well-known T cell activation molecule, and has also been implicated in chemotaxis[36], T cell development[37],

dendritic cell trafficking and humoral immune responses[38], it would be relevant to answer the question whether an intact immune response would be possible in the absence of CD38. A partial answer to this issue came from the analyses of CD38-CART cells: we remarkably discovered that the CD38-CART cells, regardless of which scFv was used, became completely devoid of CD38 expression on their surface in various independently generated batches of cells. The loss of CD38 was thus unlikely to be caused by a genetic defect, but was most probably due to the "self-lysis" of the $CD38^+$ fractions, which was also described in another CD38-CAR study[33]. Our CD38 negative CD38-CART cells, however, had no growth disadvantage, displayed highly activated status, CD38-dependent proliferation, cytokine production, cytotoxic activities and showed no other detectable functional aberrancies. This was also the case for CD19-CART cells which became CD38 negative after co-culture with CD38-CART cells (Figure S2). Furthermore CD38-CART cells did not show any defects in a transmigration assays and they also mediated significant anti-MM effects *in vivo*, thus indicating their capacity to properly migrate and infiltrate into the MM niches and to kill them. Thus, it seems likely that i) not all activated T cells have to be CD38 positive and ii) CD38 expression is not essential for T cells to fulfill their functions. This conclusion is also supported by the fact that there is yet no evidence, even in CD38 KO mice,[36] that CD38-deficient effector T cells are defective in function.

On the other hand, the relative broad expression of the target antigen of CD38-CART cells increases the risk of the so called "cytokine release syndrome (CRS)" due to massive activation of CAR-T cells, as have been observed in the previous trials with ERBB2- and CD19-CART cells.[39–41] Although the IL-6R antagonist tocilizumab appears to successfully reduce CRS[42] it would still be desirable to minimize the occurrence of such severe side effects. Furthermore, since we cannot rule out toxicities occurring die to the possible attack of non-hematopoietic $CD38^+$ cells, development of an optimal CD38-CART cell therapy would require the improvement of the target-specificity as well as the *in vivo* control on CD38-CART cells, and probably also in the case of other CART cell approaches targeting kappa light chain[43], CD138[44], Lewis Y antigen[45], BCMA[46], CS1[47,48], and CD44v6[49]. One future option to improve the target-specificity could be the optimization of the target cell affinity of CART cells. In addition, suicide genes may enable the *in vivo* control of adoptively transferred CART cells. Indeed, in our first attempt to improve the safety profile of CD38-CART cells we observed, that the inducible caspase 9 (iCasp9) gene[12,50] can effectively control CART cells. These results, which are in agreement with other studies[12,49,51] provide positive prospects for the future clinical trials. The safety profile of CART cells could also be improved by the generation of inducible CAR constructs or using the recently developed dual CAR technologies.

Taken together, we conclude that CD38-CART cells are powerful immunotherapeutic tools and can be beneficial especially for MM patients who have no other chemotherapy options. Therefore these results warrants further studies towards diminishing their undesired effects against normal $CD38^+$ cells through optimizing their CD38 affinity and improving *in vivo* controllability.

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DISCLOSURES

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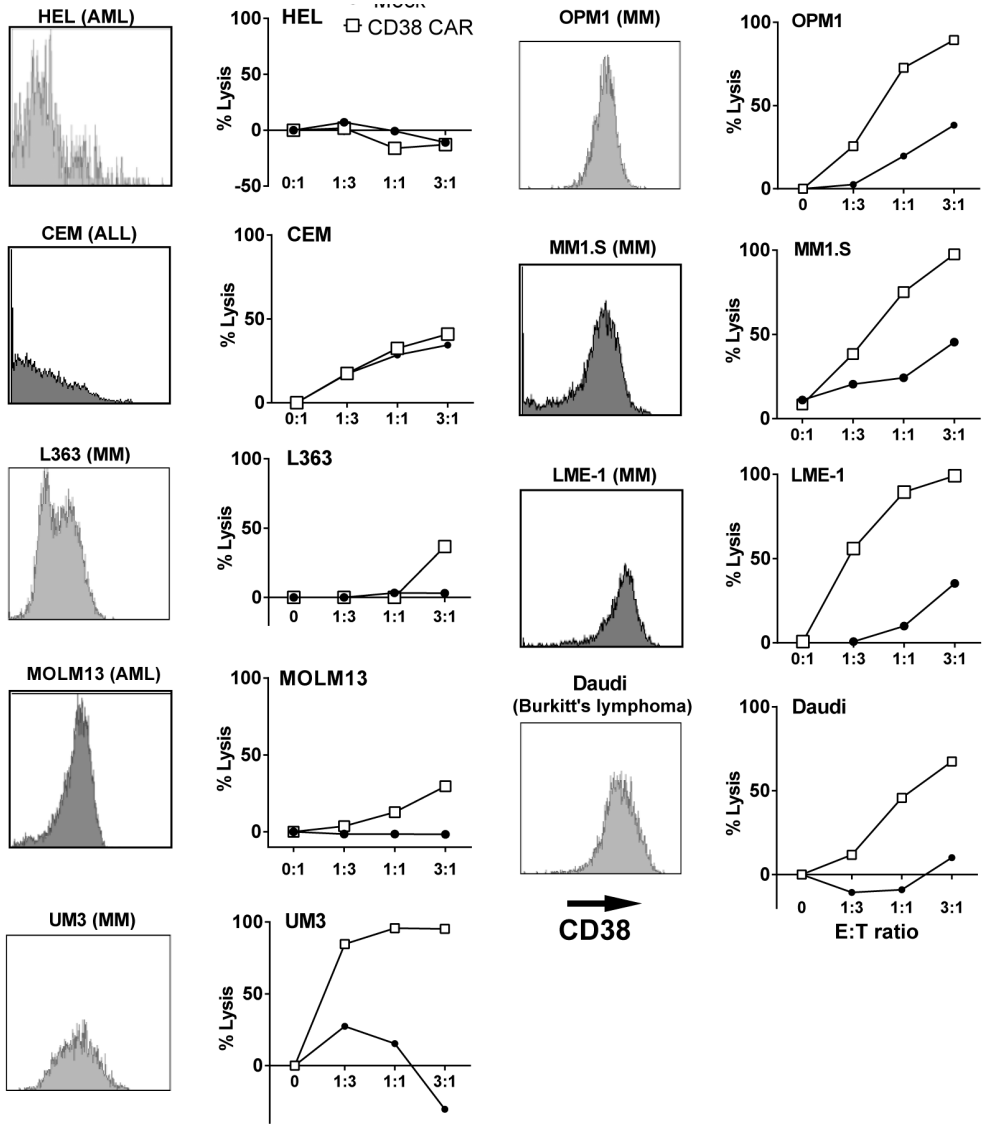
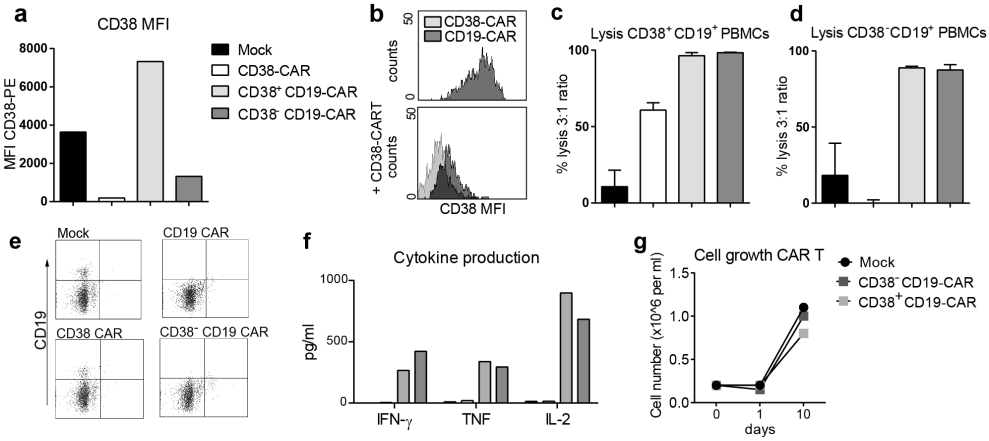
SUPPLEMENTARY MATERIAL



Supplementary figure 1. Efficacy of CD38-CAR T cells to lyse different cell line. In 24 h, cytotoxicity assays, CD38-CAR T cells (CAR056) were tested against two AML, one ALL, one Burkitt's Lymphoma and 5 MM cell lines. (A) CD38 expression histogram of cell lines, determined by flow cytometry (B) Either BLI or flow cytometry measured cytotoxicity of CD38-CAR T cells in different effector:target ratios as indicated.



Supplementary figure 2. Function analysis of CD38+ or CD38- CD19-CAR T cells. We treated the CD19-CAR T cells (with NGFR expression) with irradiated CD38-CAR T cells in a 1:1 ratio and analyzed the CD38 expression on CD38-CART cells and treated (CD38-) and untreated (CD38+) CD19-CAR T cells after 24 hours as measured with flow cytometry calculated MFI (Fig S2 A) and depicted as a histogram (Fig. S2 B; upper panel: CD19-CART alone, lower panel: CD19-CART treated with CD38-CART). The CD38 expression dropped significantly and we analyzed their capacity to eliminate CD19+ PBMCs. When incubated overnight with PBMCs, we saw a nearly 100% lysis of CD19+ PBMCs in cultures containing CD19-CART cells (Fig S2 C). The CD38-CART cells lysed ~50% of the CD19+ PBMCs, this is because roughly half of the CD19+ PBMCs were also CD38+. When solely looking at the CD19+ CD38- PBMCs, only the CD19-CAR T cells eliminated these CD19+ cells (Fig S2 D and target PBMCs plots in E). When analyzing the cytokine release upon exposure to CD19+ cell line, the CD19-CAR T cells mainly produced IFN-γ, IL-2 and TNF. This cytokine production was not significantly different between $CD38^+$ or $CD38^-$ CD19-CAR T cells (Fig S2 F). When co-incubated with previously described feeder cells (containing irradiated allogeneic PBMCs and EBV LCL cells) CD38+ or CD38- CD19-CAR T cells both expanded, similar to mock (Fig S2 G).

Supplementary table 1. Apparent affinity of selected CD38 antibodies. These CD38 antibodies were generated at Genmab BV. Indicated values are the apparent affinities (µg/ml) determined by flow cytometry binding to target cell lines CHO-CD38 and Daudi.

CD38 antibody	Affinity to CHO-CD38 cells (µg/mL)	Affinity to Daudi-luc cells (µg/mL)
3003-026	0.63	0.25
3003-028	0.32	0.11
3003-056	0.51	0.13
daratumumab	0.17	0.16

Supplementary table2. Sequences of the selected CD38 antibodies. The sequences of the variable heavy chain (VH) and variable light chain (VL) of the CD38 antibodies. Sequences listed were used to generated CARs with corresponding names.

Name	AA sequence
028	VH 028 QVQLVQSGAEVKKPGSSVKVSCKAFGGTFSSYAISWVRQAPG QGLEWMGRIIRFLGIANYAQKFQGRVTLIADKSTNTAYMELSS LRSEDTAVYYCAGEPGERDPDAVDIWGQGTMVTVSS VL 028 DIQMTQSPSSLSASVGDRVTITCRASQGIRSWLAWYQQKPEK APKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYC QQYNSYPLTFGGGTKVEIK
056	VH 056 QVQLVQSGAEVKKPGSSVKVSCKPSGGTFRSYAISWVRQAPG QGLEWMGRIIVFLGKVNYAQRFQGRVTLTADKSTTTAYMELS SLRSEDTAVYYCTGEPGARDPDAFDIWGQGTMVTVSS VL 056 DIQMTQSPSSLSASVGDRVTITCRASQGIRSWLAWYQQKPEK APKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYC QQYNNYPLTFGGGTKVEIK
026	VH 026 QVQLVQSGAEVKKPGSSVKVSCKAFGGTFSSYAISWVRQAPG QGLEWMGRIIRFLGKTNHAQKFQGRVTLTADKSTNTAYMELS SLRSEDTAVYYCAGEPGDRDPDAVDIWGQGTMVTVSS VL 026 DIQMTQSPSSLSASVGDRVTITCRASQGIRSWLAWYQQKPEK APKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYC QQYNSYPLTFGGGTKVEIK

CHAPTER 3

A RATIONAL STRATEGY FOR REDUCING ON-TARGET OFF-TUMOR EFFECTS OF CD38-CHIMERIC ANTIGEN RECEPTORS BY AFFINITY OPTIMIZATION

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ABSTRACT

Chimeric antigen receptors (CARs) can effectively redirect cytotoxic T cells toward highly expressed surface antigens on tumor cells. The low expression of several tumor-associated antigens (TAA) on normal tissues, however, hinders their safe targeting by CAR T cells due to on-target/off-tumor effects. Using the Multiple Myeloma (MM)-associated CD38 antigen as a model-system, we here present a rational approach for effective and tumor-selective targeting of such TAAs. Using the "light chain exchange" technology, we combined the heavy chains of two high-affinity CD38 antibodies with 176 germline light chains and generated ~124 new antibodies with 10 to >1000-fold lower affinities to CD38. After categorizing them in three distinct affinity classes, we incorporated the single-chain variable fragments of eight antibodies from each class into new CARs. T cells carrying these CD38-CARs were extensively evaluated for their on-tumor/off-tumor cytotoxicity as well as CD38-dependent proliferation and cytokine production. We identified CD38-CAR T cells of ~1000- fold reduced affinity which optimally proliferated, produced Th1 like cytokines and effectively lysed $CD38^{++}$ MM cells but spared $CD38^{+}$ healthy hematopoietic cells, *in vitro* and *in vivo*. Thus, this systematic approach is highly suitable for the generation of optimal CARs for effective and selective targeting of TAAs.

INTRODUCTION

Cytotoxic T cells endowed with chimeric antigen receptors (CARs) against surface antigens on tumor cells can induce powerful anti-tumor effects in experimental models and long term remissions in clinical trials. Specifically CAR T cells targeting CD19, an antigen present in B-cell leukemias and lymphomas, have shown impressive clinical results[1–5]. Hence, CAR T cells are currently considered highly appealing tools for cancer immunotherapy. Ideally the target molecule for CAR T cell therapy should be specifically expressed on tumor cells. Nonetheless, several years of research have identified only a few true tumor-specific surface antigens. Currently most tumor-associated target antigens (TAA) are expressed, albeit at low-to-intermediate levels, also on one or more normal tissues, such as epidermal growth factor receptors (EGFR and ErbB2/Her2), prostate specific membrane antigen (PSMA) or Carcinoembryonic antigen (CEA)[6,7]. Targeting such antigens with CAR T cells raises safety concerns, due to on-target off-tumor toxicities with unpredictable severity. The application of carbonic anhydrase IX–specific CAR T cells in renal cell cancer resulted in liver toxicities[8] and CEA-specific CAR T cells in colon cancer patients induced severe colitis[9]. Additionally, a high antigen load, on tumor and healthy tissues can elicit a significant cytokine response when targeted with highly reactive T cells.[3,10,11] Targeting HER2 with CAR T cells caused a fatal cytokine release syndrome (CRS), due to the recognition of low levels of HER2 expressed on the cells of lung epithelium[12].

In a recent preclinical study we have shown that CD38 is a useful target antigen for the treatment of multiple myeloma (MM) and that high affinity CD38-CAR T cells have significant anti-MM function *in vitro* and *in vivo*[13]. Although CD38 is expressed at very high levels on all MM cells, it is also present at intermediate levels on several hematopoietic cells, including NK cells, monocytes and a fraction of T cells. As expected, with high affinity CD38-CAR T cells we not only observed strong anti-MM effects, but also noted on-target off-tumor effects against normal hematopoietic cells.

Optimization of the design of the extracellular recognition domain of CARs has been proposed, among others, as an approach in order to enhance the capacity of CAR T cells to discriminate between tumors and normal tissues that express the same antigen in lower levels. Tumor selective effects of CARs have been observed when using single chain variable regions (scFv) of existing low(er) affinity antibodies[14–16]. Thus, actively decreasing and optimizing the affinity of existing antibodies could allow for minimizing the off-tumor CAR responses, which in fact has been achieved by the introduction of mutations or the replacement of human with murine residues in the scFv domain[16–19]. Nonetheless, a convenient approach that can be utilized to *de novo* generation of a large panel of candidate scFvs and methodically selection of those CARs with an optimal target affinity is still lacking.

We here describe a rational and feasible strategy, using CD38 as a model-antigen, for tumor-associated but not entirely tumor-specific antigens. To generate new antibodies binding the same epitope with a broad range of different affinities, we used the light chain

exchange technology[20–23]. Combining the heavy chains of two high-affinity CD38 antibodies with 176 germline light chains allowed the generation of more than a hundred new CD38 antibodies with 10-1000 fold lower affinities to CD38. The panel of candidate scFvs was narrowed down using rational selection criteria based on the desired immunotherapeutic properties of CAR T cells. Systematic *in vitro* and *in vivo* analysis of the newly generated CARs revealed that CAR T cells bearing scFvs derived from ±1000-fold lower affinity antibody were tumor selective killers with strong lysis of $CD38^{++}$ MM cells and little or no lysis of $CD38^{+}$ normal human hematopoietic cells.

METHODS

Antibody production by Light chain exchange

Variable heavy- and light chain coding regions were cloned in the pcDNA3.3 (Invitrogen) based vectors p33G1f and p33Kappa respectively. All 352 antibodies were produced under serum-free conditions by individually co-transfecting 2 heavy chains (024 and 028) and 176 germline light chain expression vectors in HEK-293F cells, using 293fectin (Invitrogen), as previously described[24]. A simple, robust and highly efficient transient expression system for producing antibodies[24]. Cell-free supernatants were harvested and antibody concentrations were determined by Octet IgG quantification (Forté Bio)

Bio-layer interferometry

Affinities were measured and ranked using biolayer interferometry on a Octet HTX instrument (ForteBio). Anti-Human IgG Fc Capture biosensors (ForteBio) were loaded for 1000 s with hIgG1 containing different heavy and light chain combinations directed against CD38. After a baseline (100 s) the association (1000 s) and dissociation (1000 s) of the extracellular domain of N-terminally His-tagged CD38 (His-CD38,100 nM) in Sample Diluent (ForteBio) was determined. For calculations, the theoretical molecular mass of His-CD38 based on the amino acid sequence was used, i.e. 30.5 kDa. Experiments were carried out while shaking at 1000 rpm and at 30 °C. Data was analyzed with Data Analysis software v8.0 (ForteBio), using the 1:1 model and a local full fit with 1000 s association time and 250 s dissociation time. Data traces were corrected by subtraction of the average of 4 reference biosensors loaded with IgG1-3003-028 WT and incubated with Sample Diluent only. The Y-axis was aligned to the last 5 s of the baseline, and interstep correction as well as Savitzky-Golay filtering was applied.

Antibody Binding assay

Homogenous binding assays for human CD38 specific antibodies were performed in 1536 well microtiter plates in dose response using a Tecan Evo 200 liquid handler. Binding of IgG1 antibodies to CHO cells transiently expressing human CD38, CHO wt background control and streptavidin beads coated with purified biotinylated his-tagged human CD38 was detected with a secondary polyclonal goat IgG anti-Human IgG (Fc) - Alexa Fluor

647 conjugate (Jackson ImmunoResearch). In parallel, the binding of IgG1 antibodies to streptavidin beads coated with purified biotinylated his-tagged human CD38 was also assessed using a monovalent secondary goat Fab anti-Human IgG, (H+L) - DyLight 649 conjugate (Jackson ImmunoResearch). IgG1 samples were normalized and diluted in Freestyle 293 expression medium (Gibco). Two microliter of diluted sample was added to 5 microliter cell or bead suspensions containing secondary conjugates at 200 ng/mL IgG conjugate or 300 ng/mL Fab conjugate, respectively. Cell suspensions were prepared in FMAT buffer (PBS, 0.1% BSA and 0.02% Sodium Azide) + 0.075% Pluronic F-68. Bead suspensions were prepared in HBB (10-mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 0.5% BSA and 0.01% Sodium Azide + 0.075% Pluronic F-68). After 8 hours incubation at RT in the dark, fluorescence signals were recorded using the Applied Biosystems 8200 Cellular Detection System (ThermoFisher Scientific) with a 50 counts cut-off value applied. Obtained total fluorescence intensity data was processed and visualized using ActivityBase software (IDBS).

Introduction of CD38-specific scFvs in CAR constructs

The selected variable light and heavy chains were amplified using PCR with primers (supplemental table S3) containing homology arms and Gibson assembly (NEB) was used to combine both chains linked with a G_4S linker. The generated scFvs were cloned into SFG retroviral vector, followed by a CD8a transmembrane domain and the 4-1BB and CD3ζ signaling domains as described in Zhao et al[25]. The CAR constructs were linked by a 2A sequence to a truncated NGFR or dsRed sequence[26].

Generation of retroviral particles and transduction of T cells

Phoenix-Ampho packaging cells were transfected with the CAR constructs, gag-pol (pHIT60), and envelope (pCOLT-GALV) vectors (Roche). Two and three days after transfection, cell free supernatants containing retroviral particles were collected and directly used for transduction.

Peripheral Blood Mononuclear cells (PBMCs) from healthy donors were stimulated with lectin-like phytohemagglutinin (PHA-L) in RPMI-1640 + 10% FBS. After 48 hours, cells were retrovirally transduced using spinoculation on retronectin (Takara) coated plates in the presence of Polybrene .A second transduction was done after 16 hours. 72 hours post-transduction LNGFR or dsRed and CD38 expression was determined by flow cytometry. T cells were expanded using culture medium (RPMI-1640, 10% human serum, antibiotics), and 50 IU/ml rhIL-2 (Proleukin®, Novartis). One week after, CAR-transduced T cells were either stimulated with irradiated (5 Gy) UM9 cells (E:T ratio 1:3) or tested functionally.

Primary cells from MM patients and healthy individuals

Bone marrow mononuclear cells (BMMC) containing 5-40% malignant plasma cells were isolated from bone marrow aspirates of MM patients through Ficoll-Paque density

centrifugation and either used directly or cryopreserved in liquid nitrogen until use. PBMCs/MNCs were isolated from Buffy coats of healthy blood-bank donors by Ficoll-Paque density centrifugation. All primary samples were obtained after informed consent and approval by the institutional medical ethical committee.

Cell lines

Unmodified or luciferase (Luc-GFP)-transduced human MM cell lines, UM9 and RPMI8226 were cultured in RPMI-1640 (Invitrogen) + 10% FBS (Invitrogen) + antibiotics (penicillin;100 U/ml, streptomycin; 100 µg/ml) as described.[13]

Cloning, expression and purification of CD38 extracellular domain (sCD38)

A synthetic DNA construct encoding the Honeybee Melittin signal sequence (HMSS) fused to a fragment of the extracellular CD38 domain encoding residues 43-400 was purchased (GeneArt). The HMSS-CD38 DNA was cloned into the pFastBacNKI-LIC-3C-his vector[27] to get pFastBACNKI-HMSS-CD38-3C-his (C-terminal 6xhis-tag). Bacmid DNA and virus were essentially prepared according to the Bac-to-Bac manual (Thermo Fisher Scientific). Briefly, the pFastBACNKI-HMSS-CD38-3C-his construct was transformed into EMBACY cells[28] and bacmid DNA was isolated. Sf9 insect cells were transfected with bacmid DNA and virus was harvested. Virus was amplified and used to infect sf9 cells for protein expression. Secreted proteins were harvested and concentrated in using a Fresenius Polysulfone F40S dialyzer (Fresenius Medical Care). CD38 was purified on a HiTrap Ni2+ column and eluted with 200 mM imidazol in 25 mM NaCl pH 8.0, 200 mM NaCl. CD38 was further purified by size exclusion chromatography on a S200 16/60 Superdex column (GE Healthcare) equilibrated with 25 mM NaCl pH 8.0, 200 mM NaCl buffer. The protein eluted in a single peak and fractions containing the protein were pooled and concentrated.

Flow cytometry

Flow cytometry assays were performed on FACS CantoII or LSR Fortessa (BD). Different cell subsets in T cells, PBMC or in BM-MNC in were determined using fluorescein conjugated antibodies specific for human CD3, CD4, CCR7, CD8, CD14, CD19, CD38, CD45, CD45RA, CD56, CD62L, and CD138 (BD Bioscience). Viable cells were determined with live/dead cell marker (LIVE/DEAD® Fixable Near-IR; Life Technologies L10119).

Detection of CAR expression

The sCD38 protein was used in flow cytometry experiments to determine the cell surface expression levels of high affinity CARs and to correlate to this with the expression of LNGFR, measured with an either PE or APC conjugated antibody to LNGFR (CD271) (Biolegend). An almost perfect correlation was found between the sCD38-aided measurement of CAR expression and the NGFR expression (supplemental Fig. S2). After this validation,

transduction efficiency and associated CAR expression of other CAR transduced cells was done by NGFR measurements since sCD38 did not bind to CARs with low affinity. We confirmed a comparable NGFR expression in all tested CAR T cells, the expression of the selected CARs are depicted in supplemental Fig. S3.

Flow cytometry-based cytotoxicity assay

Seven to ten days after transduction serial dilutions of CAR T cells were incubated with Violet tracer (Thermo Fisher) labeled BM-MNC or PBMC for 6-24 hours. After addition of Flow-Count™ Fluorospheres (Beckman 7547053) cells were harvested and stained for different CD markers (see above). Viable cells were then quantitatively analyzed through Flow-Count-equalized measurements. Percentage cell lysis was calculated as followed and only if the analyzed target cell population contained >500 viable cells in the untreated samples. % lysis cells = (1 − (absolute number of viable target cells in treated wells / absolute number of viable target cells in untreated wells)) × 100%.

Bioluminescent Imaging based cytotoxicity assay

To determine the lysis of Luc-GFP-transduced human malignant cell lines by CD38-CAR T cells seven to ten days after transduction., Serial dilutions of mock or CD38-CAR T cells were co-incubated with the malignant cell lines. The luciferase signal produced by surviving malignant cells was determined after 16-24 hours with a GloMax® 96 Microplate Luminometer (Promega) within 15 minutes after the addition of 125 µg/mL beetle luciferin (Promega). % lysis cells = (1 − (BLI signal in treated wells / BLI signal in untreated wells)) × 100%.

Cytokine measurement

To determine the broad array of cytokines produced by CAR T cells, we used the Cytokine Bead Array (CBA) Human Th1/Th2 cytokine kit (BD) according to manufacturer protocol. In brief, CAR T cells at 7 days post transduction were stimulated with either UM9 or U266 cells for 24 hours. Cell free supernatants were harvested and incubated with a mixture of capture beads (IL-2, IL-4, IL-5, IL-10, TNF and IFN-γ), PE-detection reagent for 3 hours. Beads were washed and analyzed by a standardized flow cytometry assay.

Hematopoietic progenitor cell growth inhibition assay

A total of 1000 $CD34^+$ sorted BM cells were mixed with effector CAR T cells at a CART:BM cell ratio of 1:1 in 0.2 mL of RPMI culture medium. After culturing for 4 hours in this small volume, the cells were resuspended to a final volume of 2 mL with semisolid Methocult (Stem cell technologies, H4534),then plated in 6cm dishes and incubated at 37°C in 5% CO_2. After 14 days, the number of colony-forming unit-granulocytes (CFU-G), and CFU-monocytes (CFU-M), were scored under a microscope.

In vivo xenograft studies

RAG2$^{-/-}\gamma c^{-/-}$ mice used in this study were originally obtained from the Amsterdam Medical Center (AMC, Amsterdam, the Netherlands) and were bred and maintained at the Amsterdam Animal Research Center. All animal experiments were approved by local ethical committee for animal experimentation and were in compliance with the Dutch Animal Experimentation Act.

We used an *in vivo* model, in which a humanized bone marrow-like environment is created in mice[29] to allow the growth of human MM tumors or normal CD34$^+$ cells in their natural niche. Briefly, hybrid scaffolds consisting of three 2- to 3-mm^3 triphasic calcium phosphate particles were coated *in vitro* with human bone marrow mesenchymal stromal cells (BM-MSC)(2×10^5 cells/scaffold). The scaffolds were implanted subcutaneously into the mice[29]. Eight to twelve weeks after implantation, mice were i.v. injected with luciferase-transduced UM9 MM cells to monitor the anti-tumor effects of CAR T cells. In a separate experiment, fluorescent (FarRed) labeled CD34+ cells were injected into the scaffold to monitor the on-target off-tumor effect of CAR T cells. After one week, when the tumors or CD34$^+$ cells became detectable by bioluminescence imaging (BLI) or Fluorescence life imaging (FLI), respectively, mice were divided in equal groups; CD38-CAR- or mock-transduced T cells (5×10^6 cells/mice) were injected by tail i.v. injections. Tumor growth or CD34$^+$ cell persistence was monitored by weekly BLI measurements. Postmortem bone marrow, spleen and scaffolds were harvested from each mouse, dissociated (spleen and scaffolds), filtered through a 70 µm filter and single cell suspensions were counted, stained and measured by flow cytometry.

Statistical methods

Statistical analyses were performed using Graphpad Prism software 6. In analyses where multiple groups were compared, either a parametric ANOVA or nonparametric Kruskal-Wallis test were used with subsequent multiple comparison. A p value <0.05 was considered significant.

RESULTS

Generation of lower affinity CD38 antibodies by light chain exchange

To generate lower affinity CD38 antibodies we applied the light chain exchange technology, in which we combined the heavy chains of high affinity antibodies 028 and 024 (described in patents WO2011154453 and WO2006099875) with 176 random germ line light chains, schematically depicted in figure 1A. One of these two antibodies, 028, was previously used to generate high affinity CD38-CAR T cells[13]. Based on previous studies, pairing of heavy chains with numerous different light chains (light chain exchange or light chain shuffling) results in the alteration of binding affinity but not epitope specificity of the antibody[21,22,30–32]. Among 352 combinations, 262 antibodies were properly expressed in sufficient quantities, and 124 of them showed binding towards CD38$^+$ transfected, but not CD38$^-$ WT CHO

cells (Fig. 1B first and fourth panel) indicating their CD38 specificity. 69 of these 124 antibodies also showed binding to recombinant CD38 coated beads. While 23 of these 69 antibodies showed bivalent binding (IgG), the remaining 46 displayed bi- and monovalent (Fab fragment) binding (quantified EC50 values for each detection method are depicted in Fig. 1C). Antibodies with the strongest binding capacity also displayed a detectable biolayer interferometry signal on an Octet HTX instrument, whereby the on-rate (k_{on}), off-rate (k_{off}) and affinity constant (K_D) (Fig. 1D and E) could be determined. As illustrated in figure 1E left panel, the K_D values of those antibodies were 10 to 1000 times lower than the K_D of the original antibody 028. Based on these data we classified all functional antibodies into three classes: Class A represented antibodies with a similar binding profile to CD38 as the parental (wildtype) antibodies but with ±10-1000 x lower affinity. Class B antibodies had no detectable affinity by biolayer interferometry but binding to CD38-positive cells/beads, (thus >1000x lower affinity to the original antibody), while the class C antibodies showed solely binding to CD38 positive cells, thus with an affinity even lower than the category B. Sequence similarities and differences in complementary determining regions (CDRs) of these antibodies are illustrated in supplemental table S1. We selected 8 antibodies from each class and generated 24 different CARs using their scFvs. A selection of soluble scFvs were evaluated for unwanted, induced aggregation or impurity, to exclude protein instability as a cause for their altered affinity (supplemental Fig. S1). PBMC-derived T cells from a healthy donor were transduced with the selected 24 newly generated CARs.

Anti-Myeloma activity of CD38-CAR T cells with variable affinities

Since the anti-tumor function of CAR T cells is of primary importance, we first determined the lytic capacity of the newly generated CD38-CAR T cells against the CD38-positive MM cell line UM9. While CAR T cells generated from class C antibodies did not lyse the UM9 cells at all, T cells transduced with CARs from Class B and A antibodies were capable of lysing MM cells. As predicted, the highest affinity CARs (class A) were better in lysing tumor cells, compared to class B (Fig. 2). Interestingly, some T cells transduced with class A antibodies (CARAx T cells) lysed the UM9 cell line as effective as the CAR T cells which were generated from the original 028 antibody (CAR028 T cells) despite their much lower affinity for CD38. On the other hand, all CARs with the 024 variable heavy chain (VH) (CARs 5 - 8 in each class) elicited inferior tumor cytotoxicity compared to CARs generated using the VH of the 028 antibody (CARs 1 - 4 in each class). Based on these results, 2 of the best CARs from both class A and B were selected (CARA1, A4, B1 and B3) (Fig. 2 indicated with arrows), and were analyzed for their proliferative capacity cytokine production and on-target off-tumor cytotoxicity to gain more insight into their immunotherapeutic properties.

Cytokine release of lower affinity CAR T cells

The selected CAR T cells were first tested for their CD38-dependent cytokine production after stimulation with MM cell line UM9. All four CAR T cells, similar to the control high

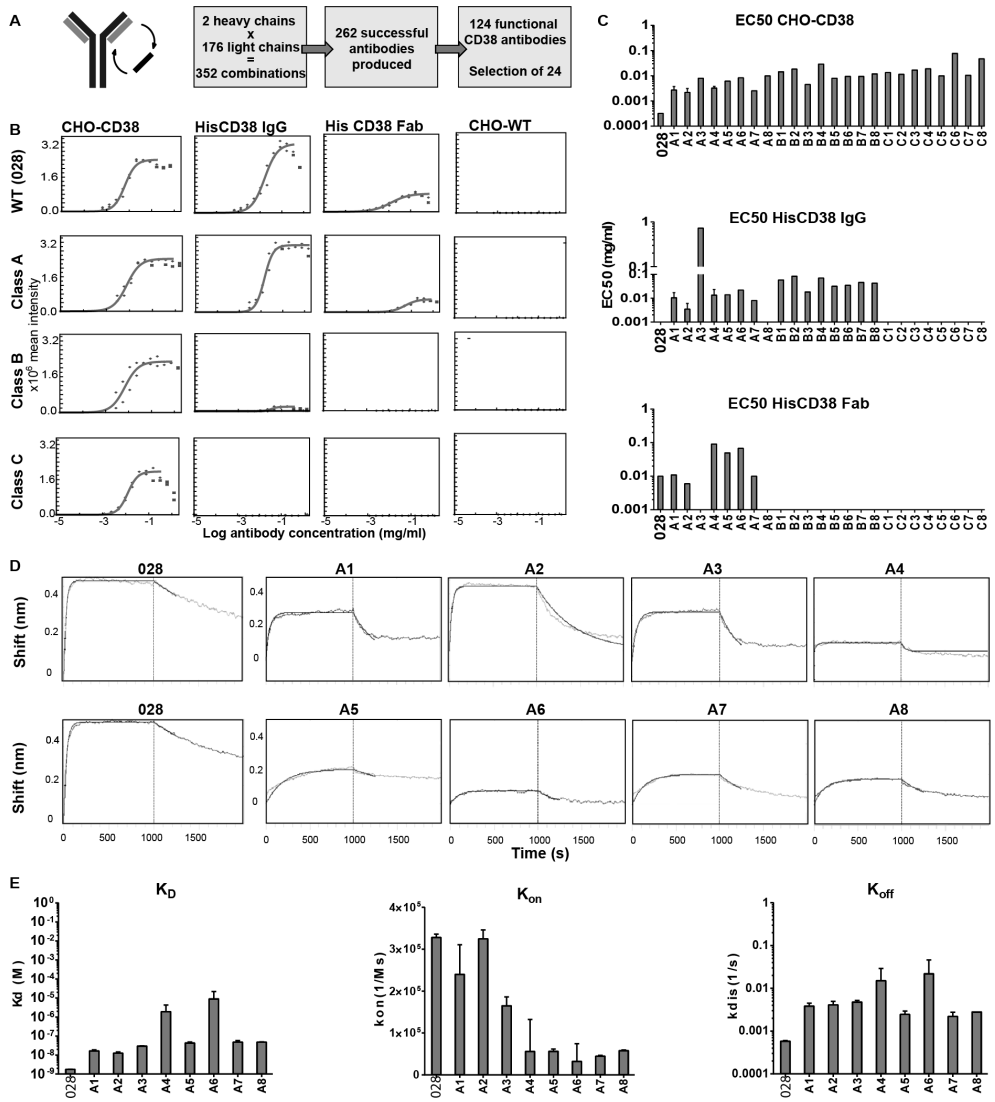

Figure 1. Selection of low affinity CD38 antibodies. (A) Schematic cartoon of light chain exchange method and overview of antibody generation and selection process. (B) Representative graphs of binding assays performed with CHO cells transfected with CD38 (CHO-CD38) or wildtype (WT). His-CD38 are beads coated with recombinant CD38 and detected with IgG or Fab fragments with fluorchrome beads. Rows indicate wildtype (WT 028) antibody 028 and class A, B and C, as defined by A= positive binding in all assays, B= positive in the cell binding and IgG, C= only positive binding on CHO-CD38. (C) Quantified EC50 values (mg/ml) of different binding assays. (D) Molecule association and dissociation curves. Interferometric profile shifts are measured and its magnitude is plotted as a function of time. (E) Interferometric profiles of the antibodies are quantified into K_D values (M), on and off-rates (Kon (1/Ms) and Koff (1/s)). N=2 +/- SD.

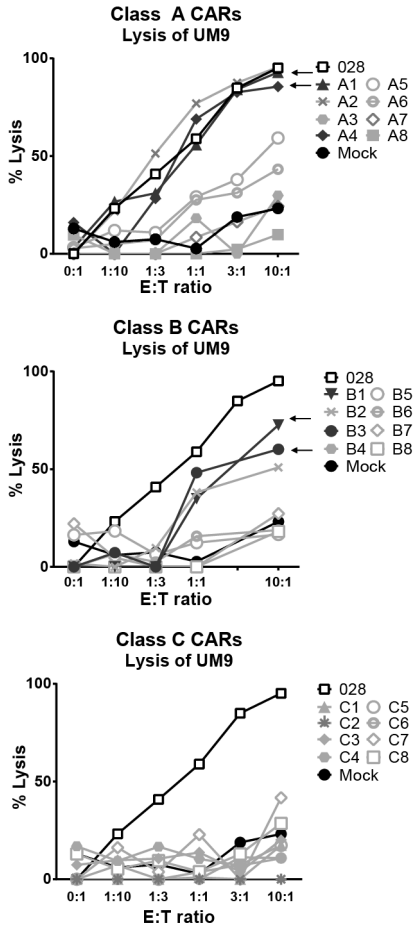

Figure 2. Lytic capacity of different affinity CD38-CAR T cells. Lysis of cell line UM9 by different affinity CD38-CAR T cells when co-incubated with luciferase-transduced MM cell line UM9 for a 16 hours, cytotoxicity was measured with BLI, n=2. Graphs are divided in three affinity subcategories. Class A CARs are derived from class A antibodies, with the highest affinity, to class C with the lowest affinity. CARs with the 028 variable heavy chain (VH) are numbered 1-4 in each class and CARs with 024 VH 5-8.

affinity CAR028 T cells, produced IFN-γ, IL-2 and TNFα in the presence but not in the absence of CD38+ target (Fig. 3A). Little or no IL-4, IL-5 or IL-10 (supplemental Fig. S4) were produced, thus indicating a typical Th1 cell phenotype. The level of cytokine production showed some association with the CAR affinity for CD38. Importantly however, the level of cytokine secretion by CARA1- and A4-transduced T cells showed no substantial difference from the high affinity CAR028 T cells.

Proliferation and expansion of lower affinity CAR T cells

We have previously showed that high affinity CD38-CAR T cells display a slower growth rate in the first two weeks due to fratricide. After this period they readily expanded, but displayed no CD38 expression on the cell surface. Therefore we also tested the CD38-dependent *in vitro* proliferative capacity and the immunophenotype of the four candidate CAR T cells after one week of transduction and after weekly stimulations with irradiated UM9 cells (Fig. 3 B and C indicated with arrows). The candidate CAR T cells displayed a similar or better growth rate compared to the control CAR028 T cells with the exception of CARA1 T cells. Furthermore the CARA4-, B3- and B1-T cells retained their CD38 expression, as opposed to CAR028 T and CARA1 T cells, (supplemental Fig. S3 right panel). Interestingly, lower affinity CAR T cells contained >50% central memory cells at the end of the production stage (1 week after transduction), similar to the mock control and in contrast to CAR028 T cells. As expected, however all cultures converted to predominantly effector memory (EM) cells after *in vitro* stimulation and expansion with tumor cells (Fig. 3C).

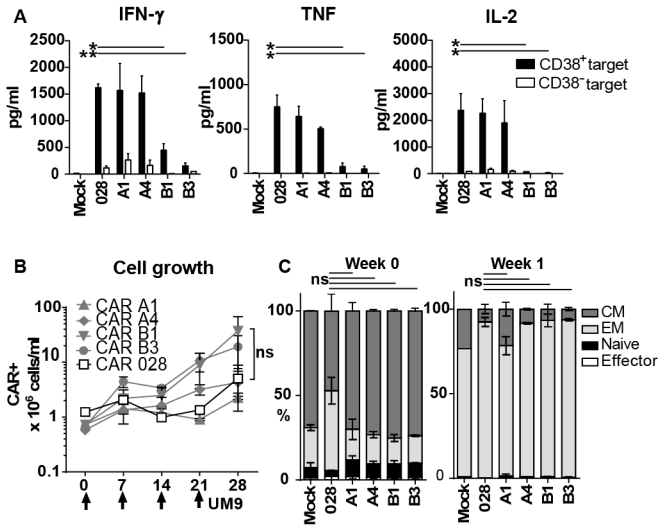

Figure 3. Phenotypic profiles of lower affinity CD38-CAR T cells. (A) 24 hours after co-incubation with CD38$^+$ target cell line UM9 or CD38$^-$ target U266, E:T ratio 1:1, cytokine secretion by mock or CD38-CAR028, A1, A4, B1 or B3 T cells was measured with a flow cytometry-based assay in the cell free supernatants. Graph shows the secretion of IFN-γ, TNF and IL-2. n=2, mean +/- SEM, * indicates p value <0.05 and ** <0.01 using one-way analysis of variance and subsequent multiple comparison. (B) CD38-CAR T cells were stimulated with MM target UM9 E:T ratio 1:3 one week after transduced and followed weekly. Cells were counted and % of CAR$^+$ cells were determined by flow cytometry. Figure indicated growth of CAR$^+$ cells in the culture. (●) indicate mock and open squares (□) indicate CD38-CAR028, (▲) CARA1, (◆) CARA4, (▼) CARB1, (●) CARB3. N=2 mean +/- SEM, ns=not significant. (C) Phenotypic profile of each CD38-CAR T cell type was determined before (week 0) and after (week 1) expansion with markers CD45RA and CD62L. Percentage of total of cells is depicted naive (CD45RA+/CD62L+) central memory (CM) (CD45RA-/CD62L+), effector memory (EM) (CD45RA$^-$/CD62L$^-$). N=2 mean +/- SEM, ns=not significant. Statistical analysis was done using one-way analysis of variance and subsequent multiple comparison.

T cells endowed with lower affinity CARs mediate no or minimal off-tumor effects

Since our specific aim was to generate CAR T cells with minimal off-tumor on-target effects by affinity optimization, we finally determined the cytotoxic activity of the four selected CARs towards MM cells as well as normal healthy cells. Although CD38 can be expressed on several tissues, we studied hematopoietic cells as the prominent candidates of off-tumor effects because preliminary immunohistochemistry assays of several normal tissues revealed that the expression of CD38 was the highest in hematopoietic cells (data not shown). For a side-by-side comparison of the on-tumor and off-tumor effects, we used primary bone marrow mononuclear cells (BM-MNCs) from MM patients as target cells, in which $CD38^{+}$ normal hematopoietic cells coexist with $CD38^{++}$ primary MM cells. Thus, after incubation of CAR T cells with BM-MNC we determined the lysis of MM cells and normal hematopoietic cells in the same sample by quantitative flow cytometry. The CAR T cells lysed $CD38^{++}$ MM cells in a roughly affinity-associated fashion ranging from 98% lysis for CARA1 to 68% lysis for the lowest affinity CARB3. (Fig. 4A top panel). Importantly, the lysis levels of CARA1 and CARA4 T cells did not significantly differ from the control CAR028 T cells

The lysis of $CD38^{+}$ fractions of normal B, T, NK cells and monocytes by CARA1, A4, B1 and B3 T cells comprised 84, 24, 23 and 6%, respectively, while the mock T cell mediated background lysis was 7%. Thus except CARA1 T cells, all other candidates showed negligible or no off-tumor effects on $CD38^{+}$ fractions of normal hematopoietic cells. When the hematopoietic cells were analyzed regardless of CD38 expression the total hematotoxicity of CARA1, A4, B1 and B3 T cells comprised only 20, 3, 20, 0%, respectively of which 11% was background, mock T cell mediated lysis. In contrast, the off-tumor effect of control CAR028 T cells was substantially higher (90% lysis of $CD38^{+}$ fraction; 39% lysis regardless of CD38 expression) (Fig. 4 B, C, illustrative flow cytometry plots in 4D). These data indicate that indeed the newly generated low affinity CAR T cells have minimal off-tumor effects.

Selection of the best lower affinity CD38-CAR T cells

Finally, to rationally select the most optimal CAR T cell, we summarized the *in vitro* data of the candidate CD38-CAR T cells, primarily according their anti-MM cytotoxicity and off-tumor hematopoietic cell toxicity, and secondarily according to cytokine production and proliferative capacity (supplemental table S2). Based on these criteria we excluded CARB3 T cells due to too low anti-tumor reactivity and CARA1 T cells due to too high off-tumor effects (Fig. 5). The remaining candidates were compared according to the secondary criteria. The CARA4 appeared the best performer since its proliferative capacity and especially the cytokine production was much better than that of CARB1.

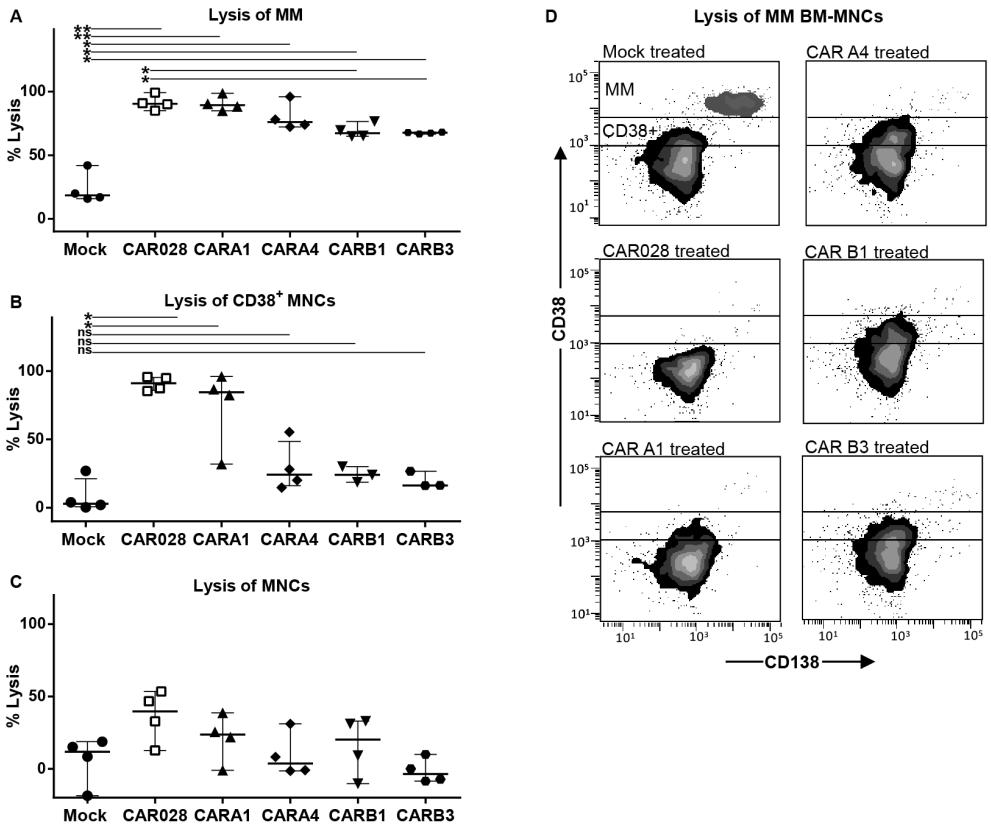

Figure 4. Lytic capacity of lower affinity CD38-CAR T cells towards Multiple Myeloma bone marrow. Bone marrow (BM-MNCs) samples of four MM patients with 20-40% MM cells were co-incubated, mock or CD38-CAR028, A1, A4, B1 or B3 T cells for 16 h. The graphs depict the resulting lysis of (A) $CD138^+/CD38^+$ cells (MM) (B) $CD38^+$ MNCs (non-MM) and (C) total MNCs in E:T (E:BM-MNC) ratio 1:1. Representative figure of other ratios in supplemental Fig. S5. (●) indicate mock and open squares (□) indicate CD38-CAR028, (▲) CARA1, (◆) CARA4, (▼) CARB1, (⬣) CARB3. The % lysis in these flow cytometry assays was calculated as described in the methods section. N=4, median +/- range, * indicates p value <0.05 and ** <0.01 using Kruskal-Wallis analysis of variance and subsequent Mann-Whitney comparison. (D) Flow cytometry dot plots depicting MM-BM with $CD138^+/CD38^{++}$ cells (MM), and $CD138^-/CD38^+$ healthy cells. The CD38 threshold for lysis is indicated with 2 horizontal bars on voltage 10^3 (threshold for high affinity CD38-CAR028) and 10^4 for some of the lower affinity CD38-CARs.

The *in vivo* anti-tumor effects of CARA4 T cells

We then evaluated the *in vivo* anti-tumor and off-tumor effects of CARA4 T cells and compared these effects with those of CAR028 and mock T cells. To mimic the human MM microenvironment, we used the specific $Rag2^{-/-}\gamma c^{-/-}$ xenograft murine model, where the luciferase transduced tumor cells are grown in humanized BM like-niches generated by subcutaneous implantation of ceramic scaffolds coated with human bone marrow stromal cells (hu-BMSCs).[29] As illustrated in figure 6A, in the negative control group treated with

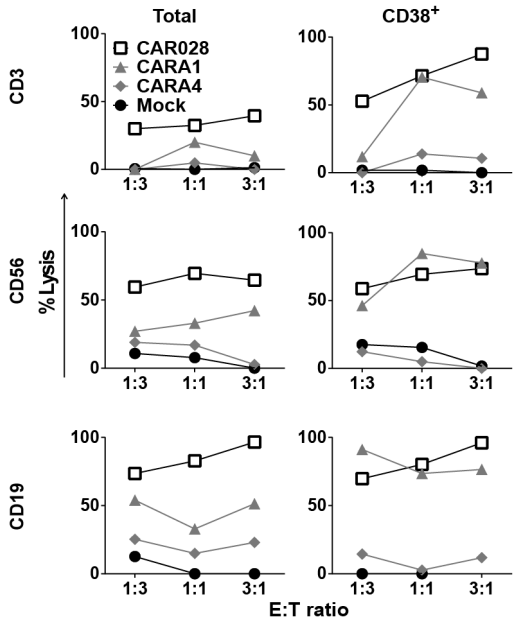

Figure 5. CARA4 is the best candidate for a lower affinity CAR. Peripheral blood mononuclear cells (PBMCs) of a healthy donor were co-incubated with mock, high affinity CD38-CAR028 or low affinity CD38-CARA4 T cells for 16 h. The graphs depict the resulting lysis of $CD3^+$ (T cells), $CD56^+$ (NK cells) or $CD19^+$ (B cells), their total or $CD38^+$ fraction. Circles (●) indicate mock and open squares (□) indicate CD38-CAR028, triangle (▲) CARA1 and diamond (◆) CARA4. The % lysis in these flow cytometry assays was calculated as described in the methods section.

mock T cells, tumors showed a fast progression. Although not curative, treatment of the tumor-bearing mice either with CAR028 or CARA4 T cells induced a significant and apparently similar anti-tumor effect (Fig. 6A, B). We did not encounter a tumor escape due to antigen-loss variants since post mortem analyses revealed that all remaining tumor cells in the mice were $CD38^+$ (supplemental Fig. S6).

The *in vivo* on target off-tumor effects of CARA4 T cells

In the *in vivo* model we observed the maximum anti-tumor effects in the first three weeks (Fig. 6). To evaluate whether the CARA4 T cells or CAR028 T cells would induce any undesired on-target off-tumor effects in this period, we injected fluorescent (FarRed) labeled $CD38^+CD34^+$ normal hematopoietic progenitor cells in the humanized scaffolds and treated the mice with i.v. injected CAR T cells. The FLI signal from normal hematopoietic progenitors was followed during 3 weeks. Injection of CARA4 T, CAR028 T or mock T cells had no effect on the FLI signal (Fig. 7A). This observation was confirmed by CAR028 and CARA4 pre-treated CD34 cells, which could still form similar numbers of colonies in a colony forming assay (supplemental Fig. S7). Nonetheless, post-mortem analysis, after three weeks revealed in mice treated with CAR028 T cells significantly lower percentages

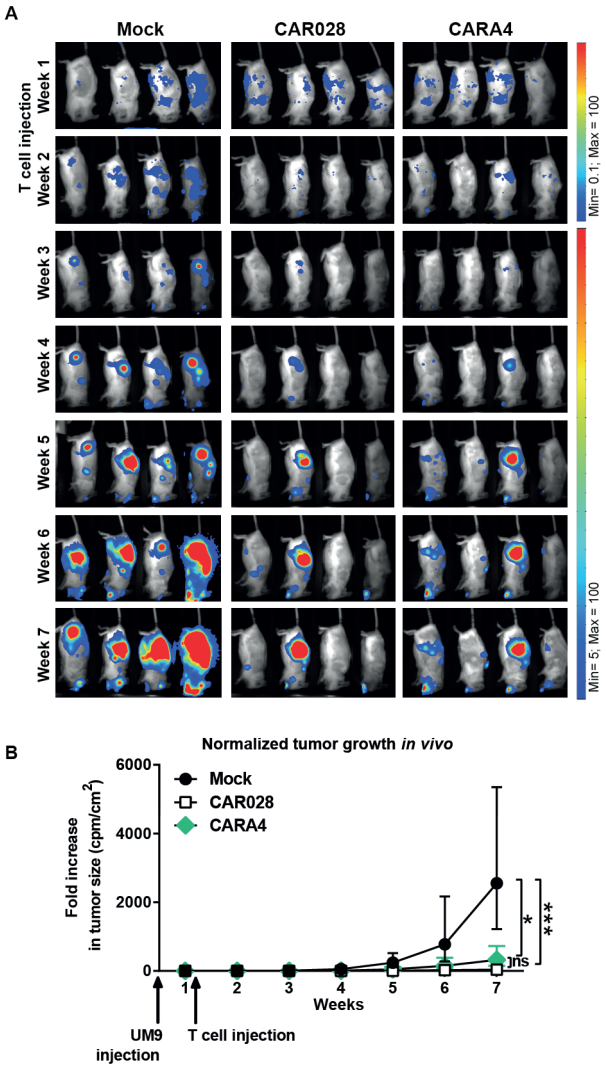

Figure 6. High and low affinity CD38-CAR T cells are similarly effective *in vivo*. Mice were i.v. injected with 10x10^6 cells of tumor cell line UM9 and treated one week after with i.v. injections of 5 x 10^6 mock, high affinity CD38-CAR028 or low affinity CD38-CARA4 T cells. (A) Bioluminescence images (BLI) are shown per group of each week. (B) Analysis of tumor load in mice by quantification of BLI measurements. Each group contained four mice, each harboring 4 scaffolds. Closed circles (●) indicate mock and open squares (□) indicate CD38-CAR028 and (◆) CD38-CARA4. N=4, Results are median tumor load (cpm/cm^2) of 4 mice +/- range, * indicates p value <0.05, ** <0.01 and *** <0.001 using Kruskal-Wallis analysis of variance.

(Fig. 7B) and total cell numbers (Fig. 7C) of CD38$^+$ cells within the CD34$^+$ fraction. Similar results were observed within the more differentiated CD34$^-$ fractions as compared to CARA4 T cell treated animals (Fig. 7B and C).

This indicated that CARA4 had no effect on CD34^{+} progenitor cells or more differentiated hematopoietic cells even if they expressed CD38. In contrast, treatment with CAR028 T cells did not hamper the total FLI signal, mediated toxicity against all CD38^{+} cells including the progenitor and differentiated cells. Since this could eventually result in defects in the differentiation of some lineages we concluded that CARA4 cells were also better performers *in vivo*.

DISCUSSION

CAR T cells have produced remarkable clinical results when targeting CD19, a surface molecule present on B-cell malignancies[33]. However, the broader application of CAR T cell therapy on the majority of cancers is limited by the simultaneous expression of TAAs - which are otherwise attractive CAR targets - on healthy tissues. Here, we propose a new feasible technique for the generation of a large panel of antibodies with different affinities. A systematic analysis and careful identification of suitable scFvs, enabled CARs that effectively target tumors with little or no off-tumor effect. We show that TAAs, such as CD38 for MM, can be selectively targeted by an scFv with an optimal affinity to the target antigen.

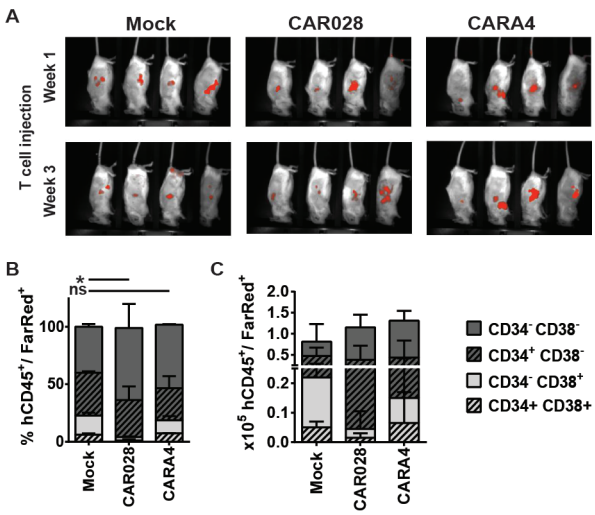

Figure 7. High affinity CD38-CAR T cells affect CD34 hematopoietic progenitor cells. (A) Mice were intrascaffold injected with 1 x 10^{6} fluorescently (FarRed) labeled CD34^{+} and treated one week after with i.v. injections of 5 x 10^{6} mock, high affinity CD38-CAR028 or low affinity CD38-CARA4 T cells. (A) Fluorescence images (FLI) are shown per group at week 1 and 3. (B) Percentages of CD34$^{+/-}$ and CD38$^{+/-}$ cells in the total population of FarRed^{+} cells, that were present in the scaffold in post-mortem tissue samples analyzed by flow cytometry. Results % CD34$^{+/-}$ and CD38$^{+/-}$ of four mice per group, two scaffold per mouse +/- range, * indicates p value <0.05, using Mann-Whitney test on CD38^{+} percentages. (C) Total number of manually counted cells were adjusted using the percentage of FarRed^{+} cells as measured by flow cytometry and the percentage of CD34$^{+/-}$ and CD38$^{+/-}$ in B. N=4 mice per group, median +/- range.

Equipping CARs with scFvs of lower affinity has been shown to avert on-target off-tumor effects and create a tumor selective window for targeting TAA with CAR T cells[14,15]. Tuning the affinity of existing antibodies by single amino acid substitutions in the scFv region[16,18] can be a useful method to create a panel of alternative CAR constructs. However, the optimal affinity range appears to differ enormously when targeting different epitopes or target antigens. Chmielewski *et al*[18] described affinities of 15-16 nM as optimal for tumor-selective ErbB2 targeting whereas Liu et al. determine an optimal antibody affinity of 1.1 nM for the same antigen. In our study, we exchanged the antibody light chain while keeping the heavy chain constant, which is suggested to modulate the affinity but not the epitope specificity of an antibody.[30,31] In contrast to previous studies, in which a limited panel of no more than 8 new scFvs was tested,[15,16,19,34] the light chain exchange technology allowed us to rapidly generate hundreds of new antibodies with a large range of affinities to the CD38 molecule. Thus, we were able to methodically identify the optimal candidate with the desired immunotherapeutic properties whereby a truly tumor-selective cytotoxic activity could be achieved. In fact our results as well as the results of others,[14–16,34] indicate that such a systematic approach is necessary because, as mentioned above, there are yet no universal parameters, which could help to predict the optimal antibody/scFv affinity for each epitope and every target antigen. Interestingly, with this technique we could lower the highest antibody affinity of 1.8 nM more than a 1000-fold and still obtain CARs with significant anti-tumor cytotoxicity and minimal off-tumor effects. While we have not tested the affinity of the scFvs to the CD38 antigen, the affinities of the selected antibodies correlated well with the CAR activity.

In order to select the optimal CAR construct we followed a logical approach in which we started with heavy chains of two high affinity antibodies (024 and 028) and extensively characterized the generated new antibodies for their affinity. We then categorized these antibodies into three "affinity groups" and selected 8 representative antibodies from each group, hereby pragmatically decreasing the pool to 24 scFv candidates to construct new CARs. The resulting 24 CAR constructs were first screened according to their anti-tumor cytotoxicity, since this is the most crucial desirable immunotherapeutic function. This approach appeared highly convenient since we could readily eliminate all antibodies within the lowest affinity class. Furthermore, although the candidate antibodies did not show significant affinity differences, the CARs generated from the 024 heavy chain were in general inferior as compared to CARs generated from 028 heavy chain, indicating the importance to start with more than one heavy chain, whenever possible. Although some 024 CARs showed intermediate lysis of MM cells, we continued only with 028-antibody based CARs, as there were already excellent candidates within this category. We extensively evaluated 4 candidates from this group which elicited >50% anti-tumor cytotoxicity in our *in vitro* assays. To select the most optimal CAR we thoroughly evaluated their several functional properties such as cytokine secretion, long-term antigen-specific proliferation, immunophenotype, but more importantly their on-target and off-tumor cytotoxicity against primary human samples. We finally selected the CARA4 as the most

optimal CAR T cells transduced with this CAR elicited similar anti-MM cytotoxic response like the original CAR028, but without having significant off-tumor toxicity on primary hematopoietic cells. Furthermore their long-term proliferation capacity was also similar to the original high affinity CAR028 T cells and they maintained Th1 cytokine production, which was even better than the other potential candidate CARB1.

To our knowledge this is the first study where CARs bearing scFvs with different antigen affinities are thoroughly tested *in vitro* and scored for all the above-mentioned properties, which define the immunotherapeutic potency of CAR T cells. Importantly, the capacity of CD38-CARA4 T cells to delay MM tumor growth in a manner similar high affinity CD38-CAR028 T cells was also confirmed in an *in vivo* murine xenograft model.

To date, most studies investigating the on-target off-tumor effects of CAR T cell therapy make the use of artificial modeling for "healthy cells" using tumor cell lines which express or are transduced to express the target antigen in low levels[15,16,35]. Although valuable in several aspects, such an approach is not ideal for evaluating the off-tumor effects of CAR T cells, since several other differences between tumor and healthy cells, especially the differences in susceptibility to cytolysis, are neglected. Therefore in our approach we always compared primary tumor cells with primary healthy cells, whose relative low proliferation rate may render them more susceptible to cytotoxicity. Moreover, in order to reduce variability and to simulate the *in vivo* clinical setting as good as possible, we executed all our *in vitro* testing of tumor cells and the healthy cells side by side, in the same compartment (BM) and at the same time. As it is suggested that results from *in vivo* models are more relevant for the potential toxicity of CAR T cell treatment,[34] we also used primary human hematopoietic progenitor cells in our *in vivo* assays. In this model, $CD34^+$ hematopoietic progenitor cells, which were inoculated in a humanized xenografted environment[13,29,36] were detectable up to three weeks in all treatment groups. This three-week evaluation period was sufficient since in the same time frame we also detected the highest level of anti-MM reactivity. Neither the high affinity CAR T cells nor the optimized affinity CAR T cells disturbed the FLI signal from the inoculated CD34 progenitor cells. However, further analyses revealed that the maintenance of the $CD38^+$ progenitor compartment was achieved only in mice treated with the low affinity CARA4, confirming the low reactivity of our lead candidate towards healthy $CD38^+$ cells.

Finally, while we have been able to optimize the antigen recognition affinity of CARs, we think that these CARs need further evaluation with respect to their signaling requirements as we have tested only CARs with a 4-1BB co-stimulatory domain. It has recently been shown that that differences in the CAR configuration, either harboring CD28 or 4-1BB co-stimulatory domains, has a large effect on the killing capacity or persistence of CAR T cells[25,37–40]. In order to retain a certain pressure on tumor cells, the co-stimulatory domains can shape the activation status of CAR T cells, as well as subsequent proliferation and cytokine production.[25,37,41] Accordingly, patient relapses seen in CAR-trials can be caused due to the poor longevity and persistence of CAR T cells. The differentiation status of the CAR T cells (central memory, effector memory or effectors) can somehow predict

the *in vivo* longevity of the CAR T cells. Interestingly, in contrast to previous studies,[14–16,34] where no difference in immunophenotype was observed when lowering the affinity of CARs, we found the maintenance of $CD38^+$ CAR T cells when using the lower affinity CARs (Supplemental Fig. S3). Furthermore, we observed an apparent higher percentage of naïve and central memory cells in lower affinity CD38-CAR T cells compared to the original CAR028 T cells at the end of production stage, after which the CAR T cells are generally injected *in vivo*. Nonetheless, all cells eventually converted to the effector memory phenotype after prolonged *in vitro* culture, suggesting that the use of other co-stimulatory domains could reveal more optimal designs for low affinity CARs.

In conclusion, our data support the feasibility of the light chain exchange method as a new approach to generate a large panel of scFvs with a wide range of affinities for a TAA epitope. We, here, propose a stepwise rational *in vitro* and *in vivo* assessment and scoring of lower affinity CAR T cells in order to identify candidates with a strong tumoricidal function and minimal off-tumor toxicity. In addition to scFv affinity, future studies should include other aspects of the CAR design such as the costimulatory moieties and the targeting of different epitopes in order to achieve optimal selective CAR T cell functionality.

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DISCLOSURES

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SUPPLEMENTARY MATERIAL

Supplementary table 1. Selected sequences of germline light chains used for light chain exchange. Sequence alignment of variable heavy and light chain. Differences in complementary determining region (CDR) 1, 2 and 3 between 028 and other light chains are highlighted in grey.

Variable heavy chains

		CDR1		CDR2		CDR3	
028	QVQLVQSGAEVKKPGSSVKVSCKAFGGTFSSYAISWVRQAPGQGLEWMGRIIRFLGIANYAQKFQGRVTLIADKSTNTAYMELSSLRSEDTAVYYCAGEPGERDPDAVDIWGQGTMVTVSS					
024	EVQLVQSGAEVKKPGESLKISCKGSGYSFSNYWIGWVRQMPGKGLEWMGIIYPHDSDARYSPSFQGQVTFSADKSISTAYLQWSSLKASDTAMYYCARHVGWGSRYWYFDLWGRGTLVTVSS					

Variable light chains

		CDR1		CDR2		CDR3	
028	DIQMTQSPSSLSASVGDRVTITCRASQGIR------SWLAWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSYPLTFGGGTKVEIK					
A1*	DIQMTQSPSSLSASVGDRVTITCRASQGIS------NYLAWFQQKPGKAPKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSYPITFGQGTRLEIK					
A2	DIQMTQSPSSLSASVGDRVTITCRASQGIS------SWLAWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSYPLTFGGGTKVEIK					
A3	DIQMTQSPSSLSASVGDRVTITCRASQSIS------SYLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGTKVEIK					
A4	DIQMTQSPSSLSASVGDRVTITCRASQGIS------NYLAWFQQKPGKAPKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSYPYTFGQGTKLEIK					
B1	EIVLTQSPDFQSVTPKEKVTITCRASQSIG------SSLHWYQQKPDQSPKLLIKYASQSFSGVPSRFSGSGSGTDFTLTINSLEAEDAATYYCHQSSSLPYTFGQGTKLEIK					
B2	EIVLTQSPATLSLSPGERATLSCGASQSVS------SSYLAWYQQKPGLAPRLLIYDASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPITFGQGTRLEIK					
B3*	AIQLTQSPSSLSASVGDRVTITCRASQGIS------SALAWYQQKPGKAPKLLIYDASSLESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQFNSYPLTFGGGTKVEIK					
B4	VIWMTQSPSLLSASTGDRVTISCRMSQGIS------SYLAWYQQKPGKAPELLIYAASTLQSGVPSRFSGSGSGTDFTLTISCLQSEDFATYYCQQYYSFPYTFGQGTKLEIK					
C1	AIQMTQSPSSLSASVGDRVTITCRASQGIR------NDLGWFQQKPGKAPKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCLQDYNYPITFGQGTRLEIK					
C2	EIVMTQSPATLSVSPGERATLSCRASQSVS------SNLAWYQQKPGQAPRLLIYGASTRATGIPARFSGSGSGTEFTLTISSLQSEDFAVYYCQQYNNWPFTFGPGTKVDIK					
C3	VIWMTQSPSLLSASTGDRVTISCRMSQGIS------SYLAWYQQKPGKAPELLIYAASTLQSGVPSRFSGSGSGTDFTLTISCLQSEDFATYYCQQYYSFPWTFGQGTKVEIK					
C4	DIQMTQSPSSLSASVGDRVTITCRASQSIS------SYLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPFTFGPGTKVDIK					
A5	EIVMTQSPATLSVSPGERATLSCRASQSVS------SNLAWYQQKPGQAPRLLIYGASTRATGIPARFSGSGSGTEFTLTISSLQSEDFAVYYCQQYNNWPLTFGGGTKVEIK					
A6	EIVMTQSPATLSVSPGERATLSCRASQSVS------SNLAWYQQKPGQAPRLLIYGASTRATGIPARFSGSGSGTEFTLTISSLQSEDFAVYYCQQYNNWPITFGQGTRLEIK					
A7	EIVMTQSPATLSVSPGERATLSCRASQSVS------SNLAWYQQKPGQAPRLLIYGASTRATGIPARFSGSGSGTEFTLTISSLQSEDFAVYYCQQYNNWPLTFGGGTKVEIK					
A8	EIVLTQSPATLSLSPGERATLSCRASQSVS------SYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPLTFGGGTKVEIK					
B5*	AIQLTQSPSSLSASVGDRVTITCRASQGIS------SALAWYQQKPGKAPKLLIYDASSLESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQFNSYPLTFGGGTKVEIK					

Supplementary table 1. (continued)

	CDR1	CDR2	CDR3
B6	DIQMTQSPSSLSASVGDRVTITCRASQGIS------NYLAWFQQKPGKAPKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSYPLTFGGGTKVEIK		
B7	DIQMTQSPSSLSASVGDRVTITCRASQGIS------SWLAWYQQKPEKAPKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSYPFTFGPGTKVDIK		
B8	DIVMTQSPDSLAVSLGERATINCKSSQSVLYSSNNKNYLAWYQQKPGQPPKLLIYWASTRESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYSTPLTFGGGTKVEIK		
C5*	DIQMTQSPSSLSASVGDRVTITCRASQGIS------NYLAWFQQKPGKAPKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSYPITFGQGTRLEIK		
C6	DIQLTQSPSFLSASVGDRVTITCRASQGIS------SYLAWYQQKPGKAPKLLIYAASTLQSGVPSRFSGSGSGTEFTLTISSLQPEDFATYYCQQLNSYPLTFGGGTKVEIK		
C7	DIQMTQSPSSLSASVGDRVTITCQASQDIS------NYLNWYQQKPGKAPKLLIYDASNLETGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCQQYDNLPLTFGGGTKVEIK		
C8	DIQMTQSPSSLSASVGDRVTITCQASQDIS------NYLNWYQQKPGKAPKLLIYDASNLETGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCQQYDNLPLTFGGGTKVEIK		

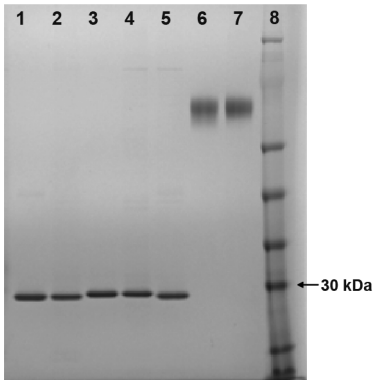
Variable light chain A1-A4, B1-B4, C1-C4 are combined with heavy chain 028. Variable light chain A5-A8, B5-B8, C5-C8 are combined with heavy chain 024.
*A5 and A7 are identical. A1 and C5, B3 and B5 are identical VLs, but form different scFvs because there are coupled to a different variable heavy chain

Supplementary table 2. Selection of the best lower affinity CD38-CAR T cells. Summary of lower affinity antibodies and CD38-CAR T cell characteristics as shown in figure 1-4. Antibody data contains the kinetics and binding determined in figure 1. Median values of on-tumor and off-tumor cytotoxicity data from figure 2 and 4. Proliferation and cytokine release as depicted in figure 3. Depicted color scale roughly indicates median values. NA=not applicable.

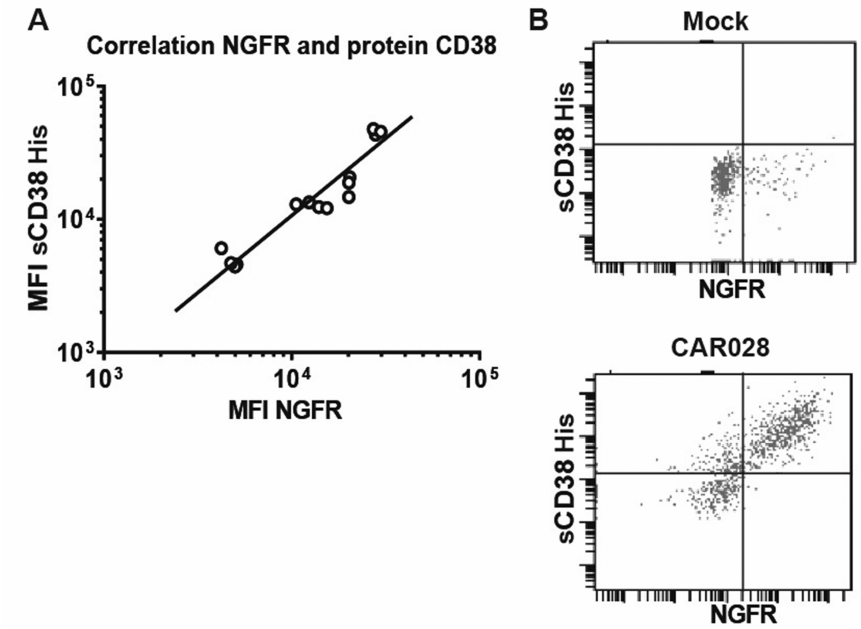
		CAR028	CARA1	CARA4	CARB1	CARB3	Mock
Antibody data	Kd value (nM)	1.8	17	1915	NA	NA	NA
	K on (1/nMs)	0.0003	0.0002	0.00006	NA	NA	NA
	K off (1/s)	0.001	0.004	0.015	NA	NA	NA
	EC50 (µg/ml)	0.3	2.7	3.3	4.3	9.8	NA
Anti-MM cytotoxicity	% lysis of MM cell line						
	% lysis of primary MM						
Off-tumor cytotoxicity	% lysis of CD38⁺ PBMCs						
	% lysis of total PBMCs						
Proliferation	Fold increase CAR⁺ cells						
Cytokines	IFN gamma						
	TNF						
	IL-2						
							
	% Lysis MM	>90%	50	<10%			
	% Lysis off-tumor	<10%	50	>90%			
	Proliferation fold increase	>2x	2x	<2x			
	Cytokines (pg/ml)	>1000	500	<100			

Supplementary table 3. Primers used for gibson assembly. 5′ – 3′ end sequences of primers used for Gibson assembly to assemble the variable heavy and light chain DNA fragments into the CAR construct. Fw= forward primer, rv= reverse primer.

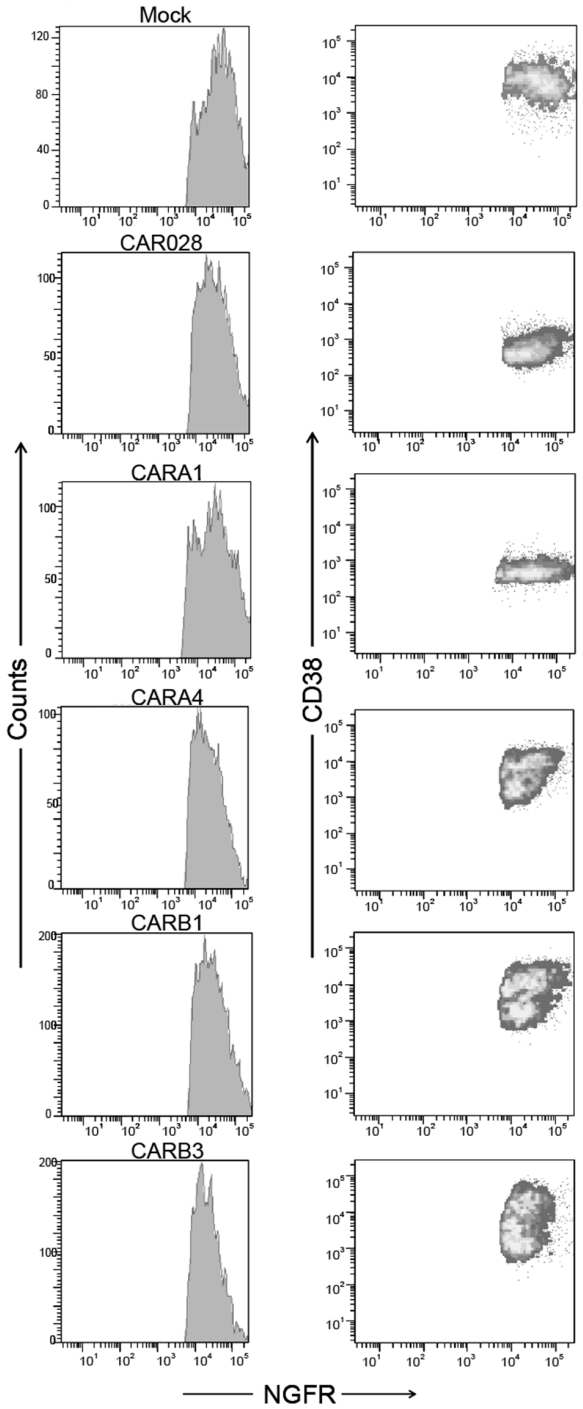
fw01	CTGCTGCTGCATGCGGCGCGCCCGGACATCCAGATGACCCAGAGC
fw02	CTGCTGCTGCATGCGGCGCGCCCGGACATCCAGCTGACCCAGAGC
fw03	CTGCTGCTGCATGCGGCGCGCCCGGCCATCCAGCTGACCCAGAGC
fw04	CTGCTGCTGCATGCGGCGCGCCCGGTGATCTGGATGACCCAGAGC
fw05	CTGCTGCTGCATGCGGCGCGCCCGGAGATCGTGCTGACCCAGAGC
fw06	CTGCTGCTGCATGCGGCGCGCCCGGAGATCGTGATGACCCAGAGC
fw07	CTGCTGCTGCATGCGGCGCGCCCGGACATCGTGATGACCCAGAGC
fw028	GCGGCGGAGGATCTGGGGGAGGCGGCTCTCAGGTGCAGCTGGTGCAGAGCG
fw024	GCGGCGGAGGATCTGGGGGAGGCGGCTCTGAAGTGCAGCTGGTGCAGTCTGG
rv01	CAGATCCTCCGCCGCCAGATCCGCCTCCGCCCTTGATCTCCACCTTGGTGCC
rv02	CAGATCCTCCGCCGCCAGATCCGCCTCCGCCCTTGATTTCCAGCTTGGTGCC
rv03	CAGATCCTCCGCCGCCAGATCCGCCTCCGCCCTTGATGTCCACCTTGGTGCC
rv05	CAGATCCTCCGCCGCCAGATCCGCCTCCGCCCTTGATTTCCAGCCGGGTGCC
rv028	GGGGCGGGTGTAGGCGGCCGCGGAGCTGGTGTTGTTGTGCTGGACACGGTGACCATTGTG
rv024	GGGGCGGGTGTAGGCGGCCGCGGAGCTGGTGTTGTTGTGCTAGACACGGTCACGAGGGTG



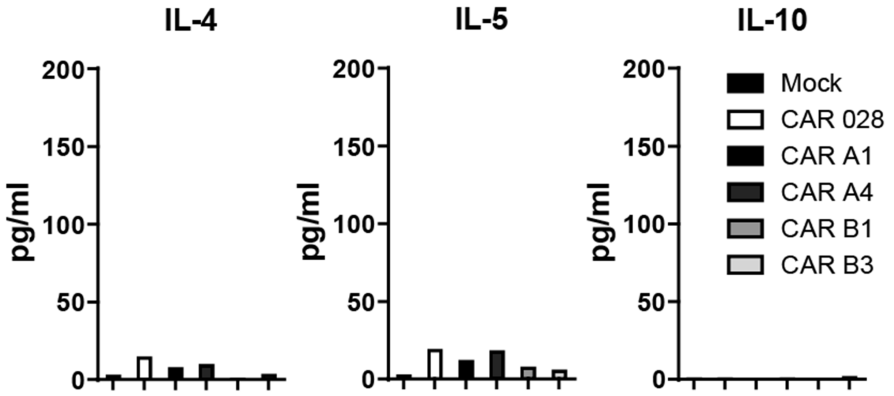
Supplementary figure 1. SDS-PAGE of single chain protein, tagged with a 6xhistidine. scFv protein was produced by 293FT-cells and purified by its His-tag. Lane 1: scFv-A1, 2: scFv-A4, 3: scFv-B1, 4: scFv-B3, 5: scFv-028. Purified protein was run on a SDS-PAGE gel. Expected size should be 28 kDa.



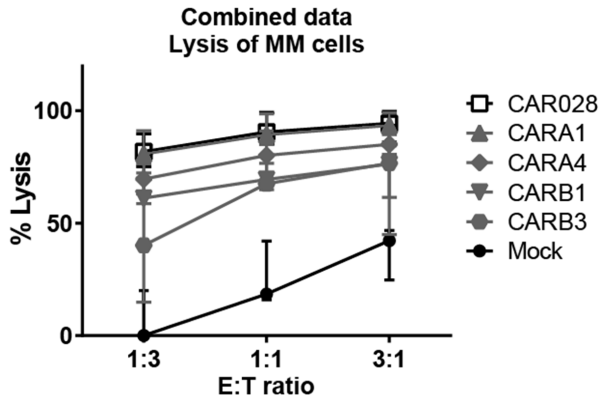
Supplementary figure 2. Validation of NGFR as surrogate marker for CAR expression. (A) Correlation between the CAR expression as measured by sCD38His with PE-labeled αHis antibody and the expression of NGFR with APC-labeled α NGFR antibody. (B) Representative flow cytometry plots of sCD38-His detection and LNGFR. Top panel Mock, bottom panel CAR028-transduced T cells.



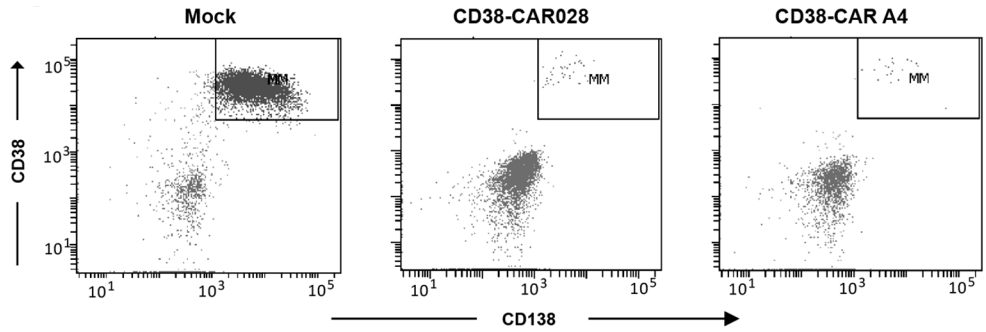
Supplementary figure 3. Expression of NGFR in CD38-CAR-2A-NGFR transduced cells. Left panel flow cytometry histogram plots with intensity of NGFR expression of NGFR$^+$ cells. Right panel density plots with LNGFR and CD38 expression as determined by flow cytometry.



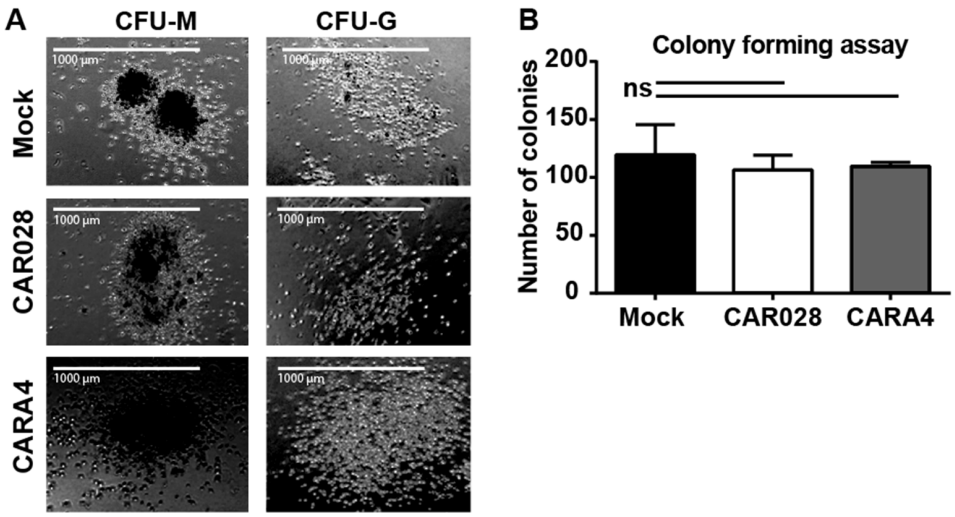
Supplementary figure 4. Cytokine profile of lower affinity CD38-CAR T cells. 24 hours after co-incubation with target cell line UM9 E:T ratio 1:1, cytokine secretion was measured with the flow cytometry-based assay in the cell free supernatants. Graph shows the secretion of IL-4, -5 and -10, which were lower than 20 pg/ml. n=2.



Supplementary figure 5. Lytic capacity of lower affinity CD38-CAR T cells towards Multiple Myeloma in the bone marrow Bone marrow derived mononuclear cells (BM-MNCs) were co-incubated, mock or CD38-CAR028, A1, A4, B1 or B3 T cells for 16 h. The graphs depict the resulting lysis of $CD138^{+}$/ $CD38^{+}$ cells (MM) , combined data from 4 independent experiments with different patient samples in E:T ratio 1:3, 1:1 and 3:1 (˜) indicate mock and open squares (£) indicate CD38-CAR028, ( ) CARA1, (¿) CARA4, (¸) CARB1, (Â) CARB3. The % lysis in these flow cytometry assays was calculated as described in the methods section. N=4, median +/- range.



Supplementary figure 6. Post-mortem analysis of scaffold harboring UM9 tumor cells. Cells were obtained from scaffolds from sacrificed mice. Single cells were stained for human CD45, CD38 and CD138 and measured by flow cytometry.



Supplementary figure 7. Colony forming assay. $CD34^+$ fraction of BM-MNCs from healthy donor was co-incubated with mock, CAR028, CARA4 T cells for 4 h at different T:BM cell ratio 1:1 before transferring into the semisolid HPC culture medium. After 14 days of culture in plastic dishes, (A) colony-forming unit-monocytes (CFU-M), and CFU-granulocytes (CFU-G) were visible, (B) the number of CFU-M, and CFU-G colonies were determined microscopically. Results of a representative experiment are shown mean + SD, ns= not significant in an unpaired standard student's t-test.

CHAPTER 4

COMBINED CD28 AND 4-1BB COSTIMULATION POTENTIATES AFFINITY-TUNED CAR T CELLS

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Under review

ABSTRACT

Targeting non-specific, tumor associated antigens (TAA) with chimeric antigen receptors (CARs) requires specific attention to restrict possible detrimental on-target/off-tumor effects. A fine-tuned reduced affinity can redirect CAR-engineered T (CAR T) lymphocytes towards high expression of TAA on tumor cells, reducing unwanted toxicities. However, CAR T cell function is affected not only by affinity but also by costimulation and it is unknown whether lowering the affinity for the target can change the costimulatory requirements of CAR T cells for optimal functionality and persistence. Using the multiple myeloma (MM)-associated CD38 antigen as a model-TAA and a series of CD38-specific scFvs having 5 different affinities, we here demonstrate the prime importance of the type of intracellular domains on the function of low affinity CAR T cells. We show that the inferior cytotoxicity and cytokine secretion mediated by CD38-CARs of very low affinity (K_D<1.9x10^{-6} M) bearing a 4-1BB intracellular domain can be significantly improved when a CD28 costimulatory domain is used. Additional 4-1BB signaling through the co-expression of 4-1BBL provided the CD38-CAR T cells with better proliferation capacity, preservation of a central memory phenotype and significantly improved *in vivo* persistence and anti-tumor function without increase of off-tumor toxicity. Hence, the combinatorial delivery of both CD28 and 4-1BB signals potentiates very low affinity CAR T cells and improves their immunotherapeutic properties. Careful affinity-tuning combined with selected costimulatory elements is the key for designing optimally effective and safe TAA-targeting CAR T cells.

INTRODUCTION

Adoptive immunotherapy with genetically engineered T cells bearing tumor-antigen specific chimeric antigen receptors (CAR) holds the potential for effective treatment of hematological malignancies and solid tumors. CARs are synthetic receptors that redirect antigen recognition and mediate T cell activation, in a single molecule, through the fusion of an extracellular antigen-binding single-chain-variable region of a monoclonal antibody (scFv) with the intracellular signaling domain from the CD3ζ chain[1]. In this way surface antigens can be recognized by CAR-engineered T (CAR T) cells independent of major-histocompatibility-complex (HLA)-mediated presentation, overcoming HLA restriction and tumor escape mechanisms. In addition, CARs endow T cells with customizable antigen recognition as scFv domains of different specificity and antigen-binding properties can be interchangeable. These properties confer a broad applicability potential to CAR T cells for a wide range of patients and diseases. Importantly, second- and third-generation CARs provide combined activation and costimulatory signals[1,2]. The addition of intracellular components from known costimulatory receptors/molecules produces signaling cascades similar to their normal counterparts and enhances T cell activation, expansion and *in vivo* persistence. Several costimulatory domains have been used in CAR design such as CD28, 4-1BB (CD137), OX-40, and ICOS either in a second- or a third-generation format[1,2]. To date, second generation CAR T cells targeting CD19 have been shown to induce impressive responses in chemotherapy resistant B cell leukemias and lymphomas (80-90% complete remissions in relapsed acute lymphoblastic leukemia). The majority of clinical studies are performed using CARs containing either CD28 or 4-1BB cytoplasmic domains[3–8].

Although CAR T cells have the theoretical potential of broad applicability for various types of tumors, their safe use requires the identification of appropriate targets, which ensure tumor eradication without off-tumor healthy tissue toxicities. CAR-target identification remains a challenge since most of the targets identified up to date are tumor-associated antigens (TAA), which are not entirely tumor-restricted and are also expressed at lower-to-intermediate levels on normal tissues. In some cases the expression of the target on healthy tissues can be tolerable and clinically manageable, such as the B-cell aplasia caused by CD19 CAR T cells[4,9], but in cases where vital tissues are involved off-tumor toxicity can be fatal[10–12]. We and others, have shown that fine-tuning the affinity of the binding domain of the CAR (scFv) can be a successful strategy to avert "on-target/off-tumor" reactivity of CAR T cells. Indeed, CARs of lower affinity targeting Erbb2/Her2, EGFRvIII or CD38 resulted in better discrimination between tumors and normal tissues expressing the same antigen in lower levels[13–16]. At the same time, decreasing the CAR affinity results in a higher target expression-threshold for T cell activation and, depending on the level of antigen expression on the tumor cells, it may also hamper the efficacy of anti-tumor function[17–19]. Previous studies that have evaluated the functionality of CAR T cells with different affinities, performed comparisons using the same CAR costimulatory design. It is, therefore, largely unknown whether lowering the affinity for the target would affect the costimulatory requirements of CAR T cells for optimal functionality and persistence.

The influence of specific costimulatory moieties on the biology and therapeutic efficacy of CAR T cells has been a subject of many recent studies. Clinical trials using either CD28 or 4-1BB-based CD19-CAR T cells have shown significant and comparable responses in patients with acute lymphoblastic leukemias[3–5,20]. However, 4-1BBζ CAR T cells seem to persist and can be detected in the circulation for longer time after CAR T cell injection, for years in some cases[21], in comparison to CD28ζ CAR T cells[3,5]. The increased longevity of 4-1BBζ CAR T cells was also corroborated in *in vitro* and *in vivo* studies and has been attributed to better maintenance of a memory phenotype and reliance on oxidative metabolism compared to CD28ζ CAR T cells[22–25]. On the other hand the addition of a CD28 costimulatory endodomain results in more IL-2 secretion and confers a more efficient and rapid cytotoxic ability to CAR T cells even at lower effector-to-target ratios[2,24]. Previous studies have shown that the combination of both synergizing signaling pathways results in greater potency and anti-tumor responses[26–28]. T cells transduced with an anti-CD19 CD28ζ-based CAR which was co-expressed with 4-1BBL (1928z-41BBL) showed the highest anti-tumor efficacy and accumulation *in vivo* and lower expression of exhaustion markers compared to other strategies aiming to deliver both CD28 and 4-1BB signaling, suggesting that this design provides the optimal combination of the two costimulatory pathways[24].

In the present study, we hypothesized that optimal CAR mediated costimulation depends on the CAR's affinity for antigen. To this end, we investigated a series of scFv's binding to the same CD38 epitope but possessing 5 different affinities[16]. We incorporated these scFvs in three different CAR designs using the 4-1BBζ or the CD28ζ, or the CD28ζ with additional expression of 4-1BBL as costimulatory elements, and evaluated the anti-tumor functionality, exhaustion phenotype, and persistence of the generated CAR T cells *in vitro* and *in vivo*. We demonstrate here that decreasing the affinity of CARs can, depending on the CAR design, compromise the anti-tumor efficacy of CAR T cells and that the combinatorial delivery of CD28 and 4-1BB signals potentiates lower affinity CARs and improves their immunotherapeutic potential *in vitro* and *in vivo* without increasing on-target/off-tumor toxicity.

METHODS

Study design

The purpose of this study was to elucidate the effect of costimulation on the immunotherapeutical characteristics of affinity-tuned CD38-CAR T cells. We previously selected 5 different scFvs, recognizing the same epitope on CD38, each with a different affinity[16],with preferential lysis of $CD38^+$ multiple myeloma (MM) cells. We designed the scFvs in either a 4-1BB-CD3z, CD28-CD3z or CD28-CD3z + 4-1BB-Ligand.

In vitro, our focus was on their cytotoxicity towards MM cell lines, primary MM cells and healthy hematopoietic cells in whole bone marrow samples. Secondary objective was the T cell cytokine production and proliferation on CD38-expressing cells, lacking exogenous cytokines and other means of costimulation. We furthermore analyzed their T

cell differentiation towards effectors and their exhaustion status, determined by checkpoint expression. In *in vivo* experiments we analyzed in separate experiments the anti-tumor and off-tumor activity of low affinity CD38-CAR T cells with a different costimulation design. The animal experiments were performed under the approval of the central authority for scientific procedures on animals (CCD). All primary samples (healthy donor and patient material) were obtained after informed consent and approval by the institutional medical ethical committee. Each experiment was performed multiple times using T cells from at least three different donors. In the figures we present pooled data (n=3) or display the data from a representative experiment.

Lower affinity CAR construction

Lower affinity CARs were produced with different germline variable light chains while keeping the variable heavy chain constant (clone 028). Selection and classification of lower affinity antibodies was described previously[16]. The selected variable heavy and light chains, separated by a G_4S linker were PCR amplified with a proofreading Q5-Hotstart polymerase (NEB) using the AfeI containing forward primer 5'ctctgctgctgcctctagcgctgctgctg3' and the NotI containing reverse primer 5'gttgtgcggccgcgctggacacggtgaccattg 3'. PCR product was purified (Bioké) and cloned into SFG retroviral vector with a T4 ligase (Roche). The scFv was followed by a CD8a transmembrane domain and the 4-1BB and CD3ζ signaling domains or a CD28 transmembrane and intracellular sequence as described in Zhao et al[24]. The CAR sequences were linked by a P2A sequence[29] to a truncated LNGFR, dsRed or 4-1BBL sequence.

Cloning of 4-1BBL

The 4-1BBL sequence was obtained form EBV-LCL cell line 10850, amplified by standard RT-PCR (Thermo Fisher) using a compatible primer pair. cDNA was used as a template to replace the dsRed, separated by a P2A from the CAR-CD28z. The forward primer including a RsrI restriction site 5'atcccggaccgatggaatacgcctctgacg3' and reverse primers with a SalI restriction site 5'ccgtcgacctattattccgacctcggtgaag 3' were used to replace the dsRed sequence with the 4-1BBL. The 4-1BBL sequence is separated by the P2A sequences and is therefore expressed separate from the CAR-28z.

Generation of retroviral particles and transduction of T cells

Phoenix-Ampho packaging cells were calcium phosphate transfected with 10 µg CAR constructs + 5 µg gag-pol (pHIT60), and 5 µg envelope (pCOLT-GALV) vectors (Roche). 16 hours post-transfection complete medium (DMEM + 10% FBS) was refreshed, and two and three days after transfection, cell free supernatants containing retroviral particles were collected and directly used for transduction.

Peripheral blood mononuclear cells (PBMCs) from healthy donors ($3x10^6$/well) were stimulated with lectin-like phytohemagglutinin (PHA-L) in a 6 well plate (Greiner Bio-One) in

culture medium (RPMI-1640, 10% FBS, penicillin;100 U/ml, streptomycin; 100 µg/ml). After 48 hours, 1 ml 3x10^6/ml of cells were transferred to retronectin coated (15 µg/ml) (Takara) 6-well plates (Falcon). Retroviral transduction was performed by addition of 2 ml virus per well followed by spinoculation (3000 rpm, 1 hour at room temperature) in the presence of 4 µg/ml Polybrene. A second transduction was conducted after 16 hours, replacing 2/3 of the cell supernatant with freshly obtained virus (2 ml). 6-8 hours after the second hit, half of the cell supernatant was replaced by fresh culture RPMI-1640 + 10%FBS and 50 IE/ml rhIL-2 (Proleukin®, Novartis) was added once. 72 hours post-transduction LNGFR, dsRed or 4-1BBL and CD38 expression were measured by flow cytometry to determine transduction efficiency.

Primary cells from MM patients and healthy individuals.

Healthy donor peripheral blood mononuclear cells (PBMCs) from buffy coats (Sanquin blood-bank) or bone marrow mononuclear cells (BM-MNCs) from MM patient's bone marrow aspirates (~10-40% malignant cells, determined by flow cytometry ($CD138^+$/$CD38^+$)), were isolated from through Ficoll-Paque (GE Healthcare Life Sciences) density centrifugation. PBS diluted blood (1:1) or bone marrow aspirates (1:5) were carefully loaded on a 15 ml ficoll layer in 50 ml tubes. A first density centrifugation step of 2000 rpm 22'30'' using no brakes, continued by cell isolation with two PBS washing steps, 10' 1600 rpm. Primary apheresis material was thawed, washed three times with PBS 10' 1600 rpm and subsequently sorted by EasySep (stem cell technologies) with CD34+ magnetic beads according to manufacturer's protocol. Isolated cells were directly used in an cytotoxicity assays or cryopreserved in liquid nitrogen until use. All primary samples were obtained after informed consent and approval by the institutional medical ethical committee.

Cell lines

The human MM cell line, UM9 (unmodified or luciferase (Luc-GFP)-transduced) was cultured in RPMI-1640 (Thermo Fisher) + 10% FBS (Invitrogen) + antibiotics (penicillin;100 U/ml, streptomycin; 100 µg/ml). The mouse fibroblast cell line NIH/3T3 cell line was obtained from ATCC and transduced with a lentivirus to express human CD38. NIH/3T3 (modified or CD38-transduced) cells and Phoenix Ampho cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher) + GlutaMAX.10% FBS (Invitrogen) and penicillin (100 U/ml) and streptomycin (100 µg/ml).

Flow cytometry

Flow cytometry assays were performed on BD LSRFortessa. Viable cells were determined with live/dead cell marker (LIVE/DEAD® Fixable Near-IR; Life Technologies L10119). Transduction efficiency and associated CAR expression was measured with an APC conjugated antibody towards NGFR (CD271) (Biolegend) for CAR-4-1BBz-LNGFR and APC antibody 4-1BBL (CD137L) (Biolegend) for CAR-28z-41BBL. CAR-28z-dsRed were

measured in the PE-CF594 channel to detect dsRed. Additional antibodies were used for weekly differentation phenotype: CD3, CD4, CD8, CD38, (BD Bioscience), CD45RA and CD62L (Biolegend), for exhaustion assays antibodies: PD-1, LAG-3 and TIM-3 (Biolegend) and for cytotoxicity assays: CD3, CD14, CD19, CD38, CD56 and CD138(BD Bioscience). To distinguish Mock/CAR T cells from target cells, target cell were stained with 0.5 μM Violet tracer (Thermo Fisher) for 25 minutes and washed before cytotoxicity assay co-cultures. Flow cytometry data analysis was performed with FACS Diva 6.1 software.

Proliferation assays

CAR T cells were counted and stimulated weekly with irradiated (50 Gy) CD38+ UM9 cells or (80 Gy) 3T3-CD38. Starting seven days post-transduction, 1×10^6 CAR^+ T cells were seeded in a 24-well plate containing 3×10^5 UM9 or 3T3-CD38 cells, to a volume of 1-1.5 ml. No additional cytokines were added and when a cell count of ~2×10^6 cells/ml was exceeded, the cell culture was split to a density of 1×10^6 per well.

Flow cytometry-based cytotoxicity assay

Seven to ten days after transduction serial dilutions (Effector:Target 3:1, 1:1 or 1:3) of CAR T cells were incubated with Violet tracer (Thermo Fisher) labeled BM-MNC or PBMC for 16-24 hours. After addition of Flow-Count™ Fluorospheres (Beckman 7547053) cells were harvested and stained for different CD markers (see section flow cytometry) to distinguish different subsets. Viable cells were then quantitatively analyzed through Flow-Count-equalized measurements. Percentage cell lysis was calculated as followed and only if the analyzed target cell population contained >500 viable cells in the untreated samples. % lysis cells = 1 − ((# viable target cells in treated wells/# of beads) / (#viable target cells in untreated wells/# of beads)) × 100%.

Bioluminescent Imaging based cytotoxicity assay

Seven to ten days after transduction serial dilutions (effector:target 10:1, 3:1, 1:1, 1:3 or 1:10) of CAR T cells were incubated with Luc-GFP-transduced human MM cell line UM9. The luciferase signal produced by surviving UM9 cells was determined after 16-24 hours with a GloMax® 96 Microplate Luminometer (Promega) within 15 minutes after the addition of 125 μg/mL beetle luciferin (Promega). % lysis cells = 1 − (BLI signal in treated wells / BLI signal in untreated wells) × 100%.

Cytokine measurements

To determine cytokine production by CAR T cells, cell supernatants were harvested 24 hours after co-culture with target cells (UM9, 3T3 or MM-BM). To measure cytokines we used Cytokine Bead Array (CBA) Human Th1/Th2/Th17 cytokine kit (BD) according to manufacturer protocol. In brief, a mixture of capture beads (IL-2, IL-4, IL-6, IL-10, IL17A,

TNF and IFN-γ), PE-detection reagent and cell supernatant were incubated for 3 hours. Beads were washed and analyzed by a BD standardized flow cytometry assay.

Intracellular phosphoprotein detection

Intracellular staining for phosphorylated signaling proteins was conducted as described by Thermo Fisher, protocol c: Two-step Protocol for Fixation/Methanol. In brief, CAR T cells were co-incubated with (violet tracer labeled) CD38+ cell line (UM9) for 0, 4, 24 or 144 hours, fixated and permeabilized with methanol. CAR+ cells were identified with dsRed or with antibodies for LNGFR or 4-1BBL and intracellularly stained with antibodies for Phospho-ZAP70/Syk (Tyr319, Tyr352) (n3kobu5), or Phospho-ERK1/2 (Thr202, Tyr204) (MILAN8R) (Thermo Fisher).

In vivo xenograft studies

$RAG^{2-/-}\gamma c^{-/-}$ mice used in this study were bred and maintained at the Amsterdam Animal Research Center. We used an *in vivo* model, in which a humanized bone marrow-like environment is created in mice to allow the growth of human MM tumors or normal CD34+ cells in their natural niche. Briefly, hybrid scaffolds consisting of three 2- to 3-mm^3 triphasic calcium phosphate particles were coated *in vitro* with human bone marrow mesenchymal stromal cells (BM-MSC)(2×10^5 cells/scaffold). The scaffolds were implanted subcutaneously into the mice[30]. Eight to twelve weeks after implantation, for the anti-tumor model 10×10^6 luciferase-transduced MM cells (UM9) were injected i.v.. Or in the separate off-tumor experiment, 1×10^6 fluorescent (FarRed) labeled healthy $CD34^+$ cells were injected transcutaneously into the scaffold. After one week, when the tumor or $CD34^+$ cells became detectable by bioluminescence imaging (BLI) or Fluorescence life imaging (FLI), respectively, mice were divided in equal groups. Mice received CD38-CAR-BBz, 28z, 28z-BBL or mock-transduced T cells (5×10^6 cells/mice), by i.v. injection in the tail vein. Tumor growth or CD34+ cell persistence was monitored by weekly BLI/FLI measurements. Postmortem, bone marrow, spleen and scaffolds were harvested from each mouse, bone marrow was flushed and spleen and scaffolds were dissociated. Flushed or dissociated tissues were filtered through a 70 μm filter and single cell suspensions were counted, stained and measured by flow cytometry.

Hematopoietic progenitor cell growth inhibition assay

A total of 2000 $CD34^+$ EasySep sorted (Stem cell technologies) cells from MM patient apheresis material were mixed with effector CD38-CAR T cells at a CART:BM cell ratio of 1:1 in 0.2 mL of RPMI + FBS culture medium. After culturing for 4 hours in this small volume, the cells were resuspended to a final volume of 2 mL with semisolid Methocult (Stem cell technologies, H4534),then plated in 6cm dishes and incubated at 37°C in 5% CO2. Between 14-21 days, the number of colony-forming unit-granulocytes (CFU-G), and CFU-monocytes (CFU-M), were scored under a microscope.

Statistical analysis

Statistical analyses were performed using Graphpad Prism software version 7.0. For normal distributions parametric student's t-tests were used. In analyses where multiple groups were compared, either a parametric ANOVA with bonferroni posthoc test or nonparametric Kruskal-Wallis test were used with subsequent multiple comparison. A p value <0.05 was considered significant.

RESULTS

Generation of CD38-CAR constructs combining different affinities and costimulation design

In order to evaluate the impact of scFv affinity changes on CAR T cells equipped with different costimulatory moieties we first generated 15 different CAR constructs through the combination of 5 scFv domains of variable affinity with 3 described CAR structural designs (Fig. 1A). We used CD38 as a model for a tumor-associated, but not tumor-specific, target since it is highly and uniformly expressed on multiple myeloma (MM) cells but is also present at lower-to-intermediate levels on subsets of healthy hematopoietic cells. We have previously generated a large panel of antibodies binding to the same epitope of CD38 with a wide range of affinities[16]. For this study, 5 scFvs were selected and ranked from high to very low affinities as depicted in Fig. 1A. These scFvs were cloned into vectors encoding the components of the two most popular second generation CAR structures providing the 4-1BB (BBz) or the CD28 (28z) modalities or a third generation combination of both. In order to deliver both 4-1BB and CD28 signaling we chose to use a design recently shown to provide the optimal combination of these two signals, where a second generation CD28ζ CAR is co-expressed with 4-1BBL (28zBBL) (Fig. 1A)[24]. The transgenes of 38BBz and 3828z CARs were linked to the functionally irrelevant markers LNGFR and dsRed respectively. Thus, the expression of LNGFR, dsRed or 4-1BBL was used as surrogate marker of CAR expression on transduced T cells, as previously validated for these constructs[16]. All generated CAR constructs were well expressed upon transduction on human peripheral blood T lymphocytes (PBL) and there was no significant difference in transgene expression levels as shown after quantification of the mean fluorescent intensity (MFI) of the respective markers (Fig. 1B and C). As expected and previously reported the high affinity CAR028 T cells expressed low levels of CD38 as a result of fratricide. In contrast, lower affinity CD38-CAR T cells showed no significant decrease of CD38 expression compared to mock transduced T cells, indicating reduced CD38-directed cytotoxicity against targets with intermediate CD38 expression ([16]and Fig. 1D)

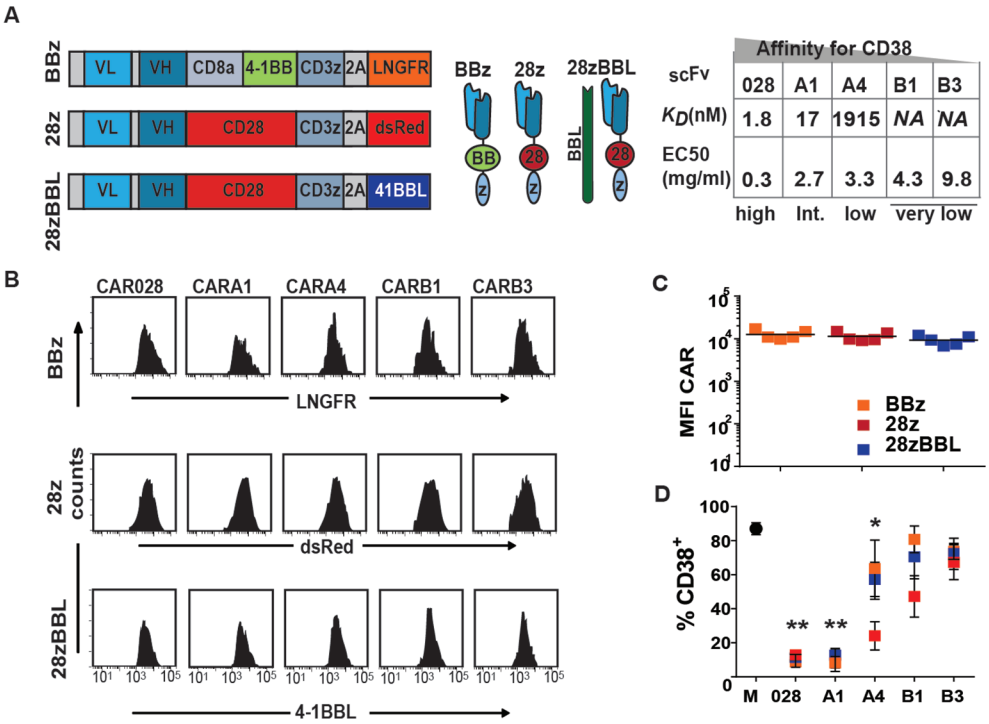

Figure 1. Schematic overview of CAR constructs and expression. (a) Overview of CAR constructs containing 4-1BB-CD3ζ-LNGFR (BBz), CD28-CD3ζ-dsRed (28z) or CD28-CD3ζ-4-1BBL (28z-BBL) (left panel) and scFvs data (right panel), the characteristics of the parental antibodies, the surface-plasmon-resonance determined K_d-value (nM) and half-effective concentration (EC50) when titrated on CHO-CD38 cells (µg/ml), described in [16]. (B) Flow cytometry histogram plots using CAR specific markers for expression LNGFR, dsRed and 4-1BBL, quantification in (C) mean fluorescent intensity (MFI) of marker expression and % CD38+ T cells when transduced with different affinity CARs. NA=not appliclable, ns=not significant, * indicates p value <0.05 and ** <0.01 using standard student's t-test analysis.

CD28 costimulation lowers the affinity threshold for efficient cytotoxicity and cytokine production

To elucidate the potential functional aberrancies caused by lowering the affinity of CD38-CAR T cells in relation to their costimulatory design, we first studied the lytic capacity of the different CD38-CAR T cell groups against the CD38-positive MM cell line UM9 (Fig. S1). In CD38-CARs carrying the 4-1BB costimulatory domain, lowering the affinity until 1000-fold (CARA4) did not significantly affect the cytotoxicity against UM9 (Fig. 2A). However, anti-tumor cytotoxicity was substantially diminished by further decrease of affinity in CARB1 and CARB3 T cells (Fig. 2A left graph). In striking contrast, when a CD28 domain was used in the CAR design, alone or in combination with 4-1BBL, the cytotoxic potential of CAR T cells with different affinities was not impacted at all. Even CARB1 and CARB3 cells, which had more than 1000-fold lower affinity for CD38, as compared to

the high affinity CAR028, displayed no significant decrease in cytotoxicity (Fig. 2A middle and right graph).

Cytotoxicity is a relatively rapid T cell response and potentially more sensitive to affinity changes. Therefore, we further analyzed the impact of an affinity decrease on the later effector functions of different CAR T cell groups, such as the cytokine secretion upon antigen encounter. Upon co-incubation with UM9 all CAR T cells produced mainly IFN-γ, TNF-α and IL-2 (Fig. 2B). The level of cytokine production was relative to the affinity for the target although no substantial effect was seen for CARs 028, A1 and A4 in all CAR-designs (Fig. 2B). On the other hand a significant decrease of all cytokines, especially TNF-α and IL-2, was observed for BBz CARs bearing very low affinity scFvs (Fig. 2B middle and right panel). Although 28z and 28z-BBL CARs also showed an affinity-dependent decrease in cytokine secretion, this effect was significant only for the CARs of the lowest affinity in our panel (Fig. 2B). Therefore, the affinity threshold for effective anti-tumor cytotoxicity and efficient cytokine production seems to be lower for CARs having a CD28 intracellular domain.

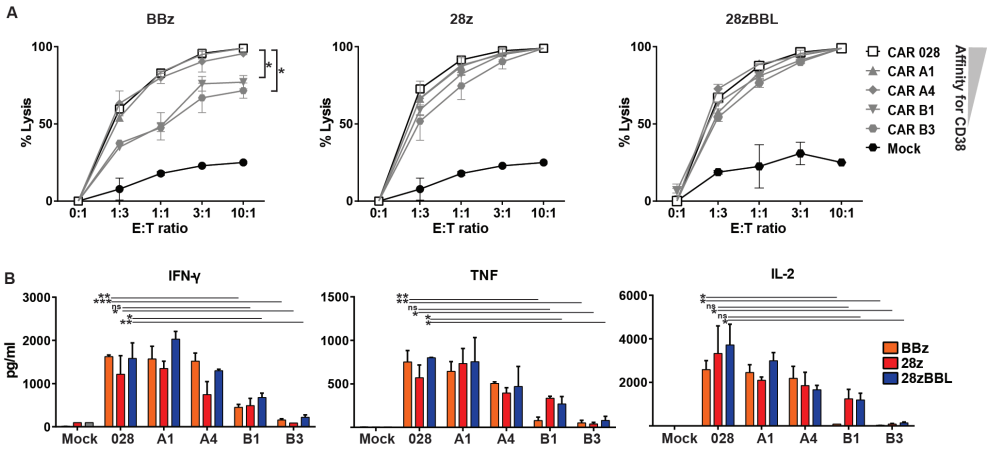

Figure 2. CD28 signaling improves lytic capacity and cytokine production of affinity-tuned CD38-CAR T cells. (A) High and low affinity CD38-CAR T cells were co-incubated with luciferase-transduced MM cell line UM9 for 24 hours. Lysis was calculated relative to untreated wells, measured by luminescence signal on a GloMax Luminometer. (□) indicate CD38-CAR028, (▲) CARA1, (◆) CARA4, (▼) CARB1, (●) CARB3. (n=3 for each condition). Left panel BBz, middle panel 28z and right panel 28z-BBL. (B) 24 hours after co-incubation with MM cell line UM9, cell supernatants were harvested to measure cytokine secretion (E:T ratio 1:1) with a flow cytometry-based assay. Graph shows the secretion of IFN-γ, TNF and IL-2. (n=3 each condition), mean +/- SEM, * indicates p value <0.05 ** <0.01 and *** <0.001 using one-way analysis of variance and subsequent multiple comparison.

Combination of CD28 and 4-1BB signaling improves *in vitro* proliferative capacity of low affinity CARs

We hypothesized that both the affinity for CD38 as well as the costimulation-induced signaling pathways could also influence the *in vitro* proliferative capacity of CARs. We, thus, weekly stimulated the CARs with irradiated NIH-3T3 cells expressing high levels of CD38 (Fig. S1) without the addition of exogenous cytokines. In this system activation and costimulation signals depends solely on the CAR binding to CD38. In our previous studies we observed that high affinity CD38-CAR T cells displayed a slower growth rate during the first week after transduction that was attributed to fratricide since CD38 is expressed in low levels on the CAR T cells themselves (data not shown). After acquiring a decreased CD38 expression on the cell surface, high affinity CD38-CAR T cells were eventually able to expand[31]. Therefore, CAR T cells were used at least after one week of culture so that fratricide would not bias the absolute number of CAR T cells as read out. Interestingly, we found that lowering the affinity for CD38 up to the level of CARB1 was slightly beneficial for the proliferation of BBz-based CD38-CARs (Fig. 3A). However, the proliferation capacity was again reduced when affinity was further lowered in CARB3-BBz cells. In all affinity groups BBz CARs showed a better proliferation potential in comparison to 28z CARs confirming previous reports[23,25,24,28,22,32], although the difference was not statistically significant (Fig. 3A). Most importantly, 28z-BBL CARs showed a stable growth response to repetitive antigen stimulations, which was always consistently higher, compared to the growth rates of both BBz and 28z CARs (Fig. 3A). Reduction of this stable proliferative response was again only seen when the CD38-CAR with the lowest affinity (CARB3) was used (Fig. 3A right panel). Therefore, CARB1 sets the threshold below which CD38-CARs of all designs showed a reduced proliferative capacity. Most importantly CD28 and 4-1BB signaling synergize for an optimal and persistent proliferative response irrespective to the CAR affinity.

4-1BB signaling and lower affinity endows CARs with a less exhausted memory phenotype

The type of costimulation can impact CAR T cell persistence by influencing their differentiation and the level of exhaustion[23,24]. In order to better interpret the proliferative capacity of the affinity-tuned CARs we further analyzed their differentiation status and the expression of exhaustion markers during expansion. Before weekly stimulation with 3T3-CD38 cells, CD38-CAR T cells were analyzed for the expression of CD45RA and CD62L (Fig. 3B). At the end of production week (week 0) CD38-CAR T cells equipped with 4-1BB signaling, either BBz or 28z-BBL, showed a significantly higher percentage of T_{CM} cells compared to 28z CAR T cells in all affinity groups and most prominently in low affinity groups A4, B1 and B3 (Fig. 3B and 3C). The same pattern was observed on CD38-CAR T cells even after 2 weeks of expansion on 3T3-CD38 cells (Fig. 3C lower panel). Interestingly, we noticed, on week 0 and less after 2 weeks, that the percentage

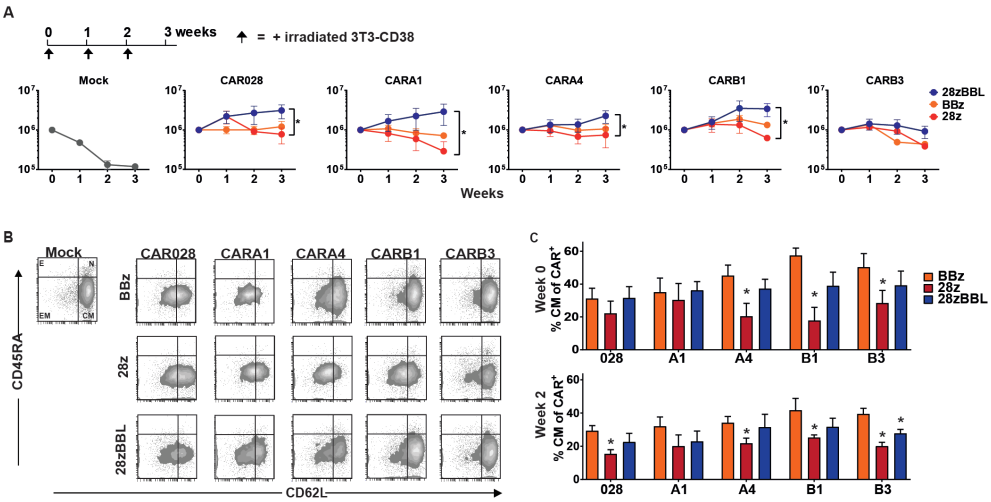

Figure 3. Combination of CD28 and 4-1BB signaling rescues in vitro proliferation and delays differentiation of affinity-tuned CD38-CAR T cells (A) CD38-CAR T cells were co-cultured with mouse fibroblast cells NIH-3T3 transduced with human CD38, E:T ratio 3:1, starting one week after transduction and re-stimulated weekly. No cytokines were added to culture. Cells were counted and % of CAR$^+$ cells were determined by flow cytometry. (B) Flow cytometry density plots of phenotypic profile of each CD38-CAR affinity and costimulation type cell type at week 0, before expansion. Cells are either of a naive (N) (CD45RA$^+$/CD62L$^+$) central memory (CM) (CD45RA$^-$/CD62L$^+$), effector memory (EM) (CD45RA$^-$/CD62L$^-$) or effector phenotype (CD45RA$^+$/CD62L$^-$). (C) Percentage of CAR+ cells is in a CM state. (n=3 for each condition) mean +/- SEM. Statistical analysis was done using one-way analysis of variance and subsequent multiple comparison, * indicates p value <0.05.

of T_{CM} cells in BBz CD38-CAR T cells increased as the affinity for CD38 decreased (Fig. 3B and C, upper panel). This was in accordance with the improvement of the growth capacity of BBz-CD38-CAR T cells when low and very low affinities were used. No further influence of affinity changes on the differentiation status of 28z or 28z-BBL CAR T cells was found.

We then analyzed the surface expression pattern of inhibitory receptors such as PD-1, TIM-3 and LAG-3 on expanded affinity-tuned CD38-CAR T cells (Fig. 4A) and determined the percentage of cells expressing none, one, two or three checkpoint markers (Fig. 4B). Interestingly, lowering the affinity for CD38 resulted in an increasing proportion of triple negative CAR T cells (expressing none of the three exhaustions markers) at week 0 irrespective of the costimulation design (Fig. 4B and Fig.S2A). This effect was at a later time point (2 weeks) prominent only for BBz and 28zBBL CARs (Fig. 4B and fig. S2A). Thus, lowering the affinity of the CAR contributed an additive effect to the maintenance of a less exhausted phenotype. Overall, BBz and 28z-BBL CD38-CAR T cells showed a lower percentage of triple positive cells (expressing all three exhaustions markers) and higher percentage of triple negative cells compared to 28z CD38-CAR T cells in all affinity groups on week 0 as well as after 2 weeks of expansion (Figure 4B and Fig. S2A). When pooling the data across all affinity groups, BBz and 28z-BBL CAR T cells showed significantly

more triple negative cells than 28z CAR T cells throughout the expansion period (Fig. S2B). These data show that, as expected, including 4-1BB signaling moieties in the CAR T cell design results in longer preservation of a central memory phenotype and a delay of the induction of immune-inhibitory receptors on the cell surface. The effect of 4-1BB costimulation in ameliorating exhaustion of CAR T cells has been previously attributed to modulation of the tonic CAR signaling[23]. However, when we assayed the basal or antigen-induced phosphorylation level of Zap70 and ERK1/2 proteins, which are main mediators of signaling downstream of CD3ζ, we found no significant difference between CARB1 cells of different designs in our system, (Fig. S3) indicating that other mechanisms could also mediate the observed phenotypic and functional differences.

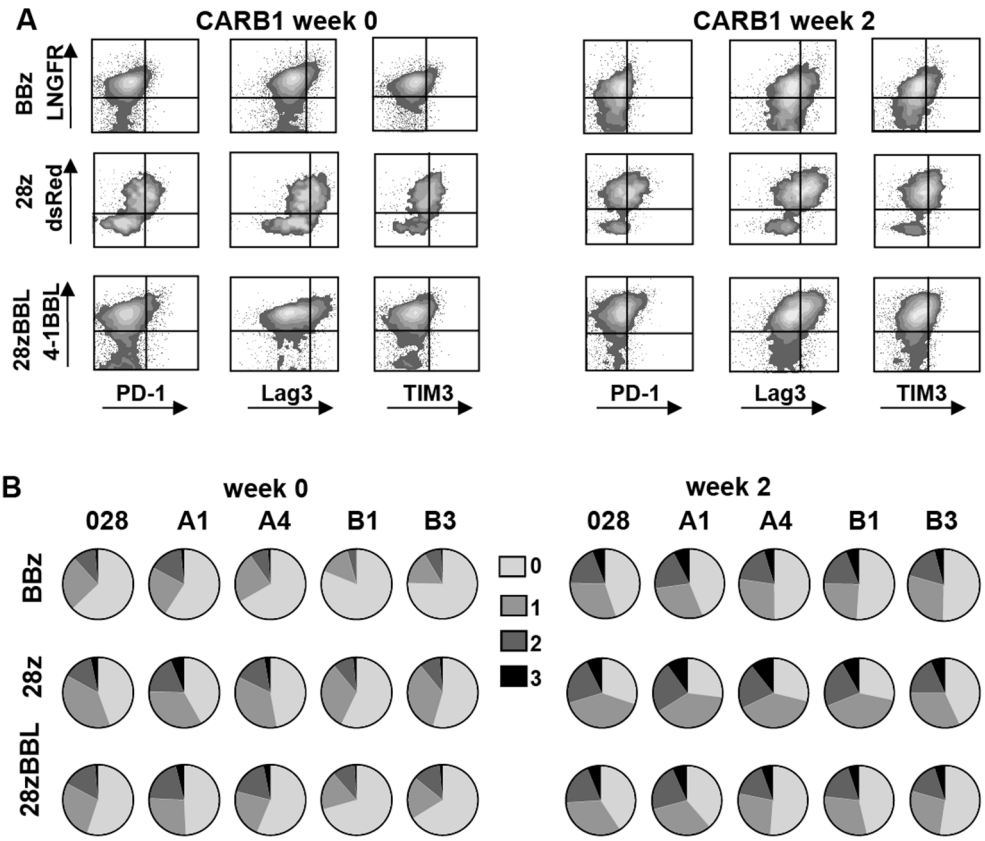

Figure 4. 4-1BB signaling delays exhaustion of affinity-tuned CD38-CAR T cells (A) Flow cytometry density plots illustrating different checkpoint marker expression of PD-1, Lag3 and TIM3, 2 weeks after stimulation with 3T3-CD38, representative figure for n=3. (B) Pie charts illustrating the % of cells expressing either 0, 1 (PD-1$^+$, Lag3$^+$ or TIM3$^+$), 2 (PD-1$^+$/Lag3$^+$ or PD-1$^+$/TIM3$^+$ or Lag3$^+$/TIM3$^+$) or 3 (PD-1$^+$, Lag3$^+$ and TIM3$^+$) checkpoint markers. Mean values of n=3.

Combined costimulation better equips very low-affinity CD38-CAR T cells to control tumor growth *in vivo*

According to our *in vitro* results, CARB1 was the most optimal lowest affinity CAR used in our study, showing an effective cytotoxic capacity similar to high affinity CAR028 when coupled to a CD28 domain and the best proliferative response when including a 4-1BB-signaling moiety. Therefore, in the further evaluation of the *in vivo* anti-MM function of lower affinity CD38-CAR T cells harboring different costimulation domains we chose to perform experiments using CARB1. The anti-MM effects of CARB1 with either a BBz, 28z or 28zBBL design were compared using a previously described model where $Rag2^{-/-}\gamma c^{-/-}$ mice are subcutaneously implanted with humanized BM like-scaffolds coated with human bone marrow stromal cells (Fig. 5A)[30,33]. Injection of $10x10^6$ UM9 tumor cells intravenously (i.v.) led to detectable tumor development in the scaffolds within the first week (Fig. 5B). At day 7, mice were i.v. injected with $5x10^6$ mock, CARB1-BBz, CARB1-28z or CARB1-28zBBL T cells (Fig. 5A). BLI imaging revealed that treatment with CARB1-28z or CARB1-28zBBL T cells resulted in a significant delay of tumor progression compared to mock treated mice while the CARB1-BBz cells failed to control tumor growth (Figure 5B, C Fig. S4). Post-mortem FACS analysis of the scaffold material, 7 weeks days after T cell treatment, showed significantly lower numbers of ($GFP^+/CD38^+/CD138^+$) tumor cells in all CAR treated groups compared to mock treated group (Fig. 5D). Therefore, it seems that CARB1-BBz T cells failed to elicit significant *in vivo* anti-MM effect due to ineffective control of tumor spread to secondary sites (e.g. murine bone marrow, skull) (Fig. S4). Specifically looking at CAR T cell numbers within the scaffolds, we found significantly higher numbers of CAR T cells persisting in CARB1-BBz and CARB1-28zBBL compared to CARB1-28z treated mice (Fig. 5E). Although CARB1-28z cells initially expanded and rapidly reduced tumor burden, they did not persist as their numbers were significantly reduced after 7 weeks (Fig. 5E and Fig. S5). On the other hand, BBz-CAR T cells showed a more delayed pattern of expansion, but they persisted for longer and eventually achieved control of tumor growth. Importantly, CARB1-28zBBL T cells displayed the best expansion and persistence features compared to the other groups as they reached the highest effector:target (E:T) ratios within the scaffolds at both an early (3 weeks) and a later (7 weeks) time point after injection (Fig. 5F and Fig. S5). In a previous study using a BBz CAR design we found that CARA4-BBz could elicit a significant anti-MM effect *in vivo*[16]. Taking into account the fact that tumor growth was similar for the mock treated group between experiments and that tumor load between treatment groups was equal before CAR T cell injection (data not shown), we normalized BLI measurements to mock control values and displayed data from both experiments (Fig. S6). Interestingly, our analysis reveals that the anti-MM activity of CD38-CAR T cells is sensitive to affinity reduction from A4 to B1 level when a BBz costimulatory design is used. Most importantly, a combined costimulation design, providing both CD28 and 4-1BB signaling, can potentiate even very-low-affinity CARB1 CAR T cells to cause significant reduction of MM tumor growth which is comparable to that obtained after treatment with high affinity CAR028-BBz CAR (Fig. S6).

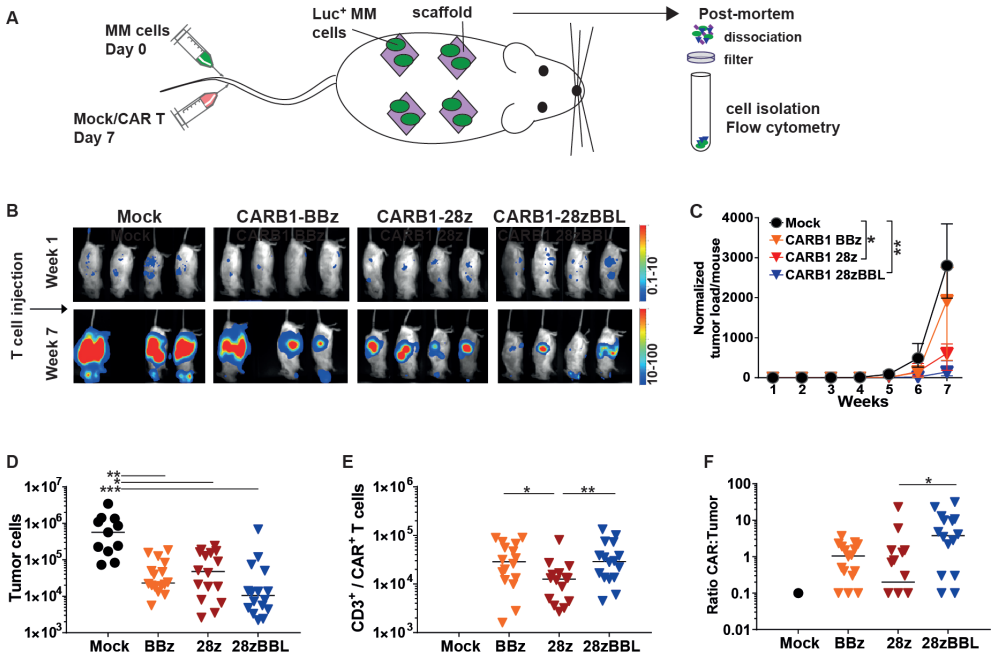

Figure 5. Very low-affinity CD38-CAR T cells with 28z and BBL costimulation show better tumor control and persistence *in vivo*. (A) Representative image of *in vivo* experimental set-up. Ceramic scaffolds coated with human mesenchymal stem cells were implanted subcutaneously 8-12 weeks before experiment. Mice were i.v. injected with 10x10^6 cells of tumor cell line UM9 and treated one week after with i.v. injections of 5x10^6 mock, CD38-CARB1 BBz, -28z or -28z-BBL T cells. (B) Bioluminescence images (BLI) are shown per group for week 1 and 7. Week 1 depicts representative image of 8 mice per group, week 7 consists N=4 per group. (all week in Figure S3) (C) Quantification of BLI measurements, normalized to week 1. Each group contained 4 mice, each mouse harboring 4 scaffolds. (N=4), Indicated are the median values of normalized tumor load per group +/- range, * indicates p value <0.05, and ** <0.01 using Kruskal-Wallis analysis of variance. (D, E, F) Post-mortem analysis of scaffold harboring UM9 tumor cells and infiltrated T cells. Cells were obtained from scaffolds from sacrificed mice. Single cells were stained for human mouse and human CD45, counted and analyzed for the % of MM cells measured by flow cytometry. (D) MM cells (GFP$^+$/CD138$^+$) (E) Mock and CAR T cells (CD45$^+$/CD3$^+$ and LNGFR$^+$ or dsRed$^+$ or 4-1BBL$^+$), Mock <10^3 T cells. (F) Ratio between Mock or CAR T cells and MM cells. N=4, results are median cell number of 16 scaffolds (4 per mouse) +/- range. Closed circles (●) indicate mock and open triangles (▽) indicate CD38-CARB1 BBz, closed triangles (▼) -28z and grey closed triangles (▼) -28z-BBL. Indicated are the median values of cell numbers and ratios per group, * indicates p value <0.05, ** <0.01, ***<0.001 using Kruskal-Wallis analysis of variance.

Combined CD28 and 4-1BB costimulation does not increase off-tumor toxicity of low affinity CAR T cells *in vitro* and *in vivo*

Since very-low-affinity CARB1-28zBBL T cells were able to elicit an anti-MM response similar to that of high affinity CAR028 *in vitro* and *in vivo*, we further investigated if that would be at the expense of increased on-target/off-tumor cytotoxicity against normal

hematopoietic cells, which are known to express intermediate-to-low levels of CD38. We therefore determined the cytotoxic activity of all CAR groups against primary MM and healthy mononuclear cells (MNCs) present in the bone marrow simultaneously in a flow cytometry-based cytotoxicity assay (>10% MM plasma cells). The observed lysis of primary MM cells had a similar pattern to that obtained against the UM9 cell line. Briefly, there was an affinity-related decrease in anti-MM cytotoxicity of CD38-CAR-BBz T cells, which was significant for CARB1 and CARB3 compared to highest affinity CAR028 (Fig. 6A left panel). When the identical scFvs were coupled to the 28z costimulatory domain (+/- 4-1BBL) the overall anti-MM cytotoxicity was improved, confirming our previous results. As expected, the anti-MM activity of CARB1 and CARB3-28zBBL was not significantly different from that of CAR028-28zBBL (Fig. 6A middle and right panel). Reducing the affinity in the CD38-CAR-BBz T cells resulted in a very limited lysis of healthy mononuclear cells (MNC) which was not significantly different from control (mock) T cells for CARA4, CARB1 and CARB3 confirming our previous reported data[16] (Fig 6B left panel, different E:T ratios are depicted in Fig. S7). On the other hand, CD38-CAR-28z +/- BBL T cells showed an increased cytotoxicity towards healthy MNCs, even for CARA4 (Fig. 6B middle and right panel). This indicated that CARA4, which has 1000-fold lower affinity than CAR028 and a K_D value in the micromolar range, mediates a higher off-tumor toxicity when coupled to a 28z construct, while its BBz counterpart appears safe. Thus, to prevent unwanted cytotoxicity of 28z-bearing CARs, an even more reduced affinity was required. Importantly, the very-low-affinity CARB1 and CARB3, either in a 28z or 28zBBL format, did not elicit significant lysis of healthy MNCs despite their effective anti-MM cytotoxic response (Fig. 6B middle and right panel).

We next evaluated the on-target off-tumor toxicity of CARB1-28zBBL T cells against human $CD34^+CD38^+$ progenitor cells *in vivo*. To this end, we used a previously established model where fluorescent (FarRed) labeled $CD34^+$ normal hematopoietic progenitor cells were injected in the humanized scaffolds and one-week later mice received i.v. treatment with CAR T cells[16] (Figure 7A). The FarRed labeled cells could still be detected by their FLI signal 14 days after CAR T cell injection in all treatment groups. Thus, treatment with either high or low affinity CD38-CARs left $CD34^+CD38^-$ stem cells intact and did not inhibit hematopoiesis (Fig. 7B) as also demonstrated by the limited cytotoxicity on $CD34^+$ stem cells observed in a colony forming assay (Fig. S8). Post-mortem flow cytometric analysis of scaffold contents revealed, as expected, that treatment with high affinity CAR028-28zBL T cells resulted in significant decrease of the percentage of $CD38^+/CD34^+$ (Fig. 7C) and total $CD38^+$ cells (Fig. 7D) compared to mock treated controls. However, low affinity CARB1-28zBBL T cells, similar to CARA4-BBz cells, caused no damaging effect on $CD34^+/CD38^+$ cells. Therefore, equipping very-low-affinity CD38-CARs with a combined CD28 and 4-1BB costimulatory signaling moieties can improve their cytotoxic function without inducing off-tumor cytotoxicity of healthy cells expressing lower levels of the target antigen.

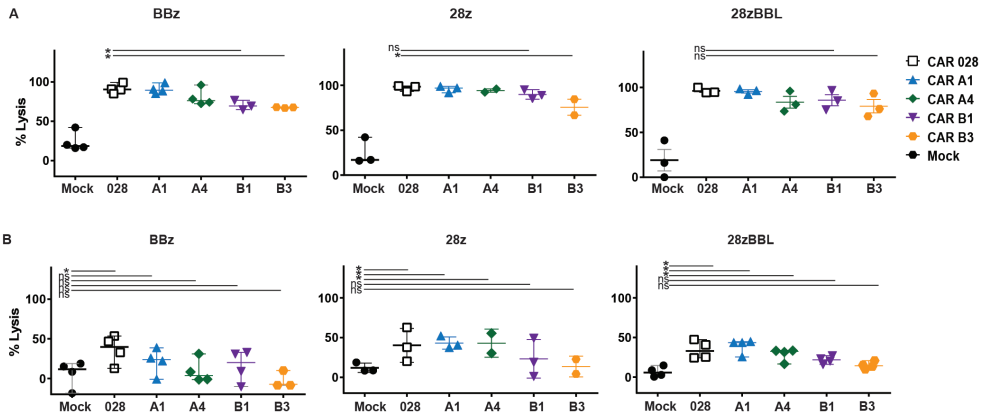

Figure 6. On-tumor and off-tumor effects of affinity-tuned CD38-CAR T cells. Bone marrow (BM-MNCs) samples obtained from 2-4 MM patients with 20-40% MM cells were co-incubated with, mock or CD38-CAR028, A1, A4, B1 or B3 T cells for 16 h. The graphs depict the resulting lysis of (A) CD138⁺/CD38⁺ cells (MM) and (B) total MNCs in E:T (E:BM-MNC) ratio 1:1. The % lysis in these flow cytometry assays was calculated as described in the methods section. (□) indicate CD38-CAR028, (▲) CARA1, (◆) CARA4, (▼) CARB1, (●) CARB3. n=3 or 4 for each condition, median +/- range, * indicates p value <0.05 and ** <0.01 using Kruskal-Wallis analysis of variance and subsequent Mann-Whitney comparison.

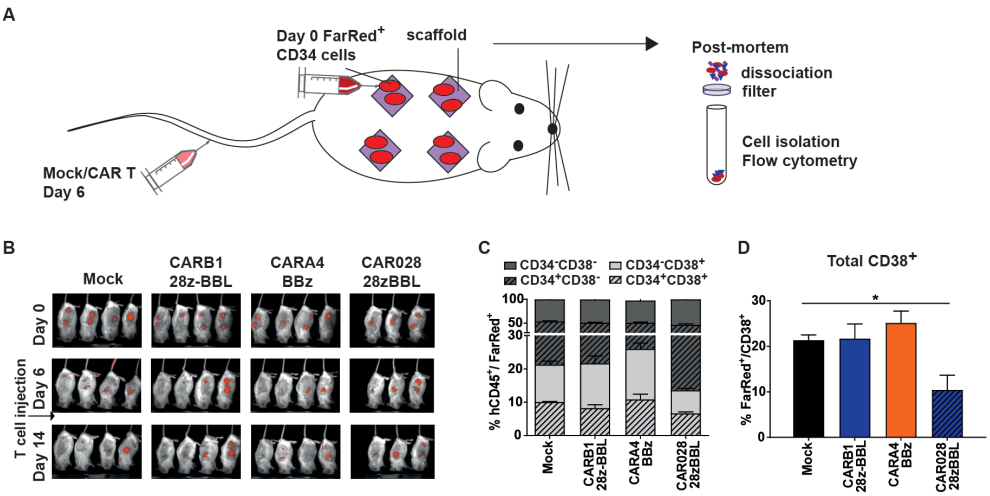

Figure 7. Very low-affinity CD38-CAR T cells with 28zBBL design do not lyse healthy CD38+ hematopoietic cells *in vivo*. (A) Mice were injected intrascaffold with 1 x 10^6 fluorescently (FarRed) labeled CD34+ and treated 6 days later with i.v. injections of 5 x 10^6 mock, high affinity CAR028 28z-BBL, or low affinity CARA4 BBz or CARB1-28z-BBL T cells. (B) Fluorescence images (FLI) are shown per group at week 1, 2 and 3. (B and C) Post-mortem scaffold samples analyzed by flow cytometry. (C) Percentages of CD34+/- and CD38+/- cells and (D) percentage of total CD38 in the total population of FarRed⁺ cells within the scaffold. N=4 mice per group, median +/- range. Results % CD38+ of four mice per group, two scaffold per mouse +/- range, * indicates p value <0.05, using Mann-Whitney test on CD38+ percentages

DISCUSSION

The applicability of CAR T cell therapy beyond B cell malignancies is in part impeded by safety concerns about the on-target/off-tumor effect of CAR T cells on normal tissues expressing low levels of the target antigen. Adjusting the affinity of CAR T cells to target antigen density is one popular strategy to avert "on-target/off-tumor" toxicity and confer specificity to the tumor[16,14,15,13]. In addition, lowering the affinity of the CAR may influence and improve toxicities related to cytokine release[34]. However, CAR T cell activation and function is a multifactorial process, which is affected not only by affinity but also by avidity and costimulation. Here, we investigated the impact of costimulatory CAR design when fine-tuning the CAR affinity. We indeed show that CARs with different costimulatory designs, providing CD28 or 4-1BB signaling or both, respond differently to stimulation through high or low affinity scFvs.

In this study, CD38 was used as a paradigm of tumor-associated antigen (TAA), as it is expressed in high levels on MM tumor cells but also in lower levels on healthy hematopoietic cells. We used a previously described panel of CD38-targeting CARs bearing scFv domains spanning a wide range of affinities for the target[16]. For a specific affinity, CAR T cell activation and cytotoxic response is relative to the level of expression of the target and lowering the affinity increases the target-expression threshold for effective CAR T cell activation[18,17,35,19,36]. Therefore, the lower the target affinity of the CAR, the better the capacity to discriminate between tumor and healthy cells with lower target expression. Importantly, our scFv panel not only covered high and low affinity levels similar to the ones used in other studies[14,15,18,35] (K_D ranging between $1.8x10^{-6}$ M-$1.9x10^{-9}$ M), but also included and investigated scFvs from antibodies whose K_D could not be determined and their binding to the target was only measurable in cell-binding assays. These scFv domains were coupled to CD28 or 4-1BB intracellular signaling domains in order to construct the most commonly used second generation CARs. Based on a study by Zhao *et al.*[24] we coexpressed a 28z-based second generation CAR with 4-1BBL as an effective conformation to provide CAR T cells with both CD28 and 4-1BB signaling. It is well demonstrated that costimulatory moieties derived from CD28 or 4-1BB or a combination of the two provide different biological and metabolic characteristics to CAR T cells leading to differences in anti-tumor lytic capacity, differentiation and persistence[2,25,24,32]. However all these comparative studies have been performed using CARs bearing binding scFv domains with high affinity against the target antigen.

Our data revealed that this different cell programming, mediated by costimulation, affects also the efficiency of both early and later CAR T cell responses when scFvs of lower affinity are used. We show that, when using very low affinities in the micromolar range (K_D $<1.9 \times 10^{-6}$ M), 4-1BB-based CD38 CAR T cells began to lose their capacity for anti-MM lysis and cytokine production (such as IL-2 and TNF-α). This indicated that 4-1BB-based CAR designs are more sensitive to lowering the affinity to the antigen than 28z-based CARs. Previous studies evaluating the targeting of ErbB2 and EGFRvIII positive

tumors with low affinity CARs using a 4-1BB intracellular domain reported no significant loss of cytotoxicity or cytokine production against tumor cells[14,15]. However, the scFvs with the lowest affinities tested in these studies (K_D values of $1.1x10^{-9}$ M for ErbB2 and $1.01x10^{-7}$ M for EGFRvIII) were still not as low as our very-low-affinity scFvs and those scFvs of our panel having similar affinities (028, A1 and A4) also showed no reduction of cytotoxicity. Interestingly, we found that inclusion of CD28 signaling reduced the affinity threshold for efficient activation after antigen encounter and rescued cytotoxicity and cytokine production in very-low-affinity CAR T cells. It has been demonstrated that CD28 can lower the threshold for effective TCR activation and can enhance responses in cases of low antigen availability (low avidity)[37,38] but its effect in cases of lower TCR affinity is not known. Our data are in contrast with Chmielewski *et al.* [35], who reported an affinity threshold of 10^{-8}, same affinity level as CARA1, below which CD28 signaling did not improve activation of ErbB2-CAR T cells. This discrepancy could be explained by the fact that the dissociation rate described in Chmielewski *et al.*[35] for ErbB2-scFv with K_D $<10^{-8}$ was 10-fold lower than that of our low affinity CARs. It seems that affinity fine-tuning by keeping a low dissociation rate results in longer interaction of the CAR with the antigen and ensures efficient and potent CAR activation.

Furthermore, affinity tuning had an effect on the proliferation and the differentiation of CD38-CARs especially for the designs including 4-1BB signaling (BBz and 28z-BBL). We found that lowering the affinity for CD38 improved slightly the proliferative response and increased the prevalence of T_{CM} cells of CD38-BBz CAR T cells as well as decreased exhaustion of both CD38-BBz and CD38-28zBBL CAR T cells. While not statistically significant and restricted to *in vitro* experiments, this observation is in line with studies showing superior anti-tumor efficacy of low affinity CARs and TCRs[39–42]. Moreover, it is known that the natural range of affinities for T cell receptors (TCR) for their interactions with pMHC molecules is between 1-100 $x10^{-6}$ M in order to achieve optimal T cell activation and function[39,41]. The use of transgenic TCRs displaying affinities beyond this range resulted in faster exhaustion of T cells and poor *in vivo* anti-tumor outcomes[43,44]. Our data in combination with these studies indicate that lowering the affinity of CARs, especially those designs providing 4-1BB signaling, could not only serve to avoid "on-target/off-tumor" toxicity but also could be a choice for optimizing CAR persistence and anti-tumor function.

Irrespective to the CAR affinity, our data confirmed previous studies showing different biological properties between CAR T cells bearing CD28 or 4-1BB costimulatory signaling domains[23,25,24,28]. For all different affinities in our study 4-1BB-based CAR T cells showed less rapid differentiation, less exhaustion and better proliferative capacity *in vitro* than CD28-based CAR T cells, during the production period as well as after sequential antigenic challenge. Similar to Zhao *et al.*[24] we found that both BBz and 28z CD38-CAR T cells restricted MM cell growth within the scaffolds *in vivo*, although they did so by following different kinetics. However, CD28-based CAR T cells resulted in better control of total tumor growth than BBz CARs and they showed more rapid and efficient tumor elimination even in lower E:T ratio. Supplying full CAR costimulation triggers both pathways, which

results in a more balanced T cell stimulation[24,28,45,46]. Indeed, our in *vitro* and *in vivo* results confirm that addition of 4-1BBL expression to a 28z-based CAR combines optimally the two signaling pathways potentially by regulating downstream signaling of the CD3ζ-domain, in addition to other proposed mechanisms[24,28].

We demonstrate that the CD28z+4-1BBL configuration is an ideal design to provide low affinity CD38 CAR T cells with enhanced anti-tumor cytotoxic potential (through CD28 signaling) and ameliorated proliferative capacity, retention of a memory phenotype and reduced exhaustion (through 4-1BB/4-1BBL signaling) *in vitro*. Furthermore, in our *in vivo* xenograft murine MM model, treatment with very-low affinity CAR T cells (CARB1) having a 28zBBL design resulted in superior restriction of tumor growth and CAR T cell expansion and persistence as compared to any of the second generation CD38-CAR designs. Most notably, comparative analysis with previous *in vivo* data revealed that a 28zBBL design could potentiate even very-low affinity CAR T cells to elicit anti-tumor responses comparable to that obtained by CAR T cells with >1000 times higher affinity for the target. Clinically most relevant is the fact that this potentiation of anti-tumor function did not compromise the safety of very-low affinity CARs. When tested against healthy $CD38^+$ hematopoietic cells CARB1-28zBBL cells showed no significant "off-tumor" toxicity *in vitro* as well as *in vivo*. Especially in the whole primary BM cytotoxicity assay the CARB1-28zBBL cells were able to successfully eliminate primary MM tumor cells while sparing surrounding healthy hematopoietic cells. Although optimal affinity levels for different individual target molecules cannot be compared, to our knowledge this is the lowest affinity for a TAA-targeting CAR that has been shown to be both efficient and safe. Our findings suggest that even scFvs with practically unmeasurable K_D values and obtained from monoclonal antibodies, which would otherwise be discarded, can be effective when used for CAR T cell therapy if optimal costimulation is provided.

The present analysis focuses on the well-described and most frequently used 4-1BB and CD28 signaling domains. Nevertheless, there are other costimulatory domains that have been used in the design of CARs such as OX40, CD40L and ICOS. The OX40, like 4-1BB belongs to the TNFR family and primarily signals through the NF-kB pathway[47,48]. On the other hand, CD40L and ICOS are CD28-like and signal through PI3K pathway[48–50]. Although 4-1BB and CD28 can be considered representatives of the two receptor families, a different combination of costimulatory moieties may result in similar findings. A consideration in our study is the fact that CD38 is expressed in T cells resulting in significant fratricide when high-affinity CARs were used. Taking into account this limitation we performed functional assays after one week of culture when we assured that no further fratricide takes place and we considered this week as the first antigen encounter for the CAR T cells when investigating differentiation phenotype and exhaustion.

In conclusion, we demonstrate here that the selection of the costimulatory design of CARs is of critical importance when using scFv domains with very low affinity for the target. We show that, if equipped with an optimal combination of CD28 and 4-1BB costimulatory moieties, CARs bearing antigen-binding domains with K_D values even lower than 10^{-6} M

can elicit significant anti-tumor cytotoxic and proliferative response without compromising their safety. The results of this study unlock the use of very-low-affinity CARs in order to target TAAs, potentially increasing the capacity to discriminate between tumor and healthy cells and suggest that careful construction of TAA-targeting CARs will enhance their clinical potential.

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DISCLOSURES

There is no relevant potential conflict of interest to disclose.

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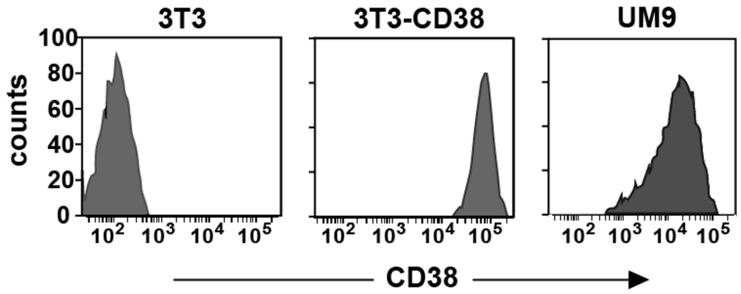
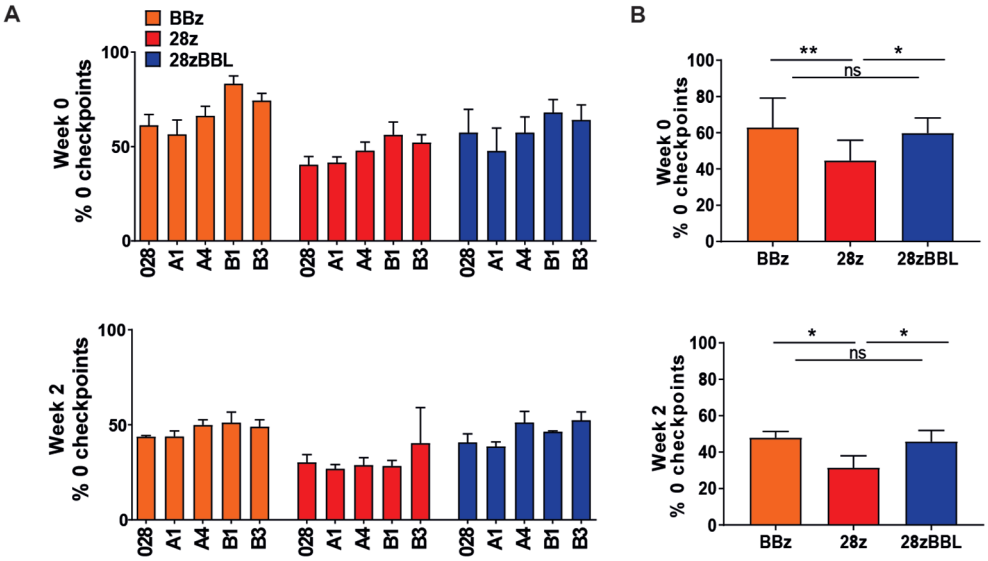
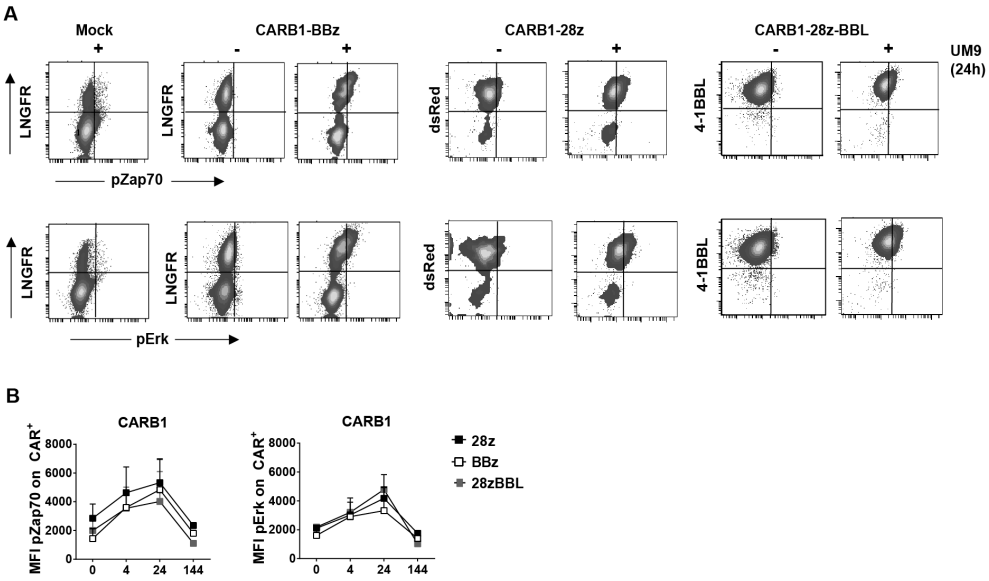
SUPPLEMENTARY MATERIAL



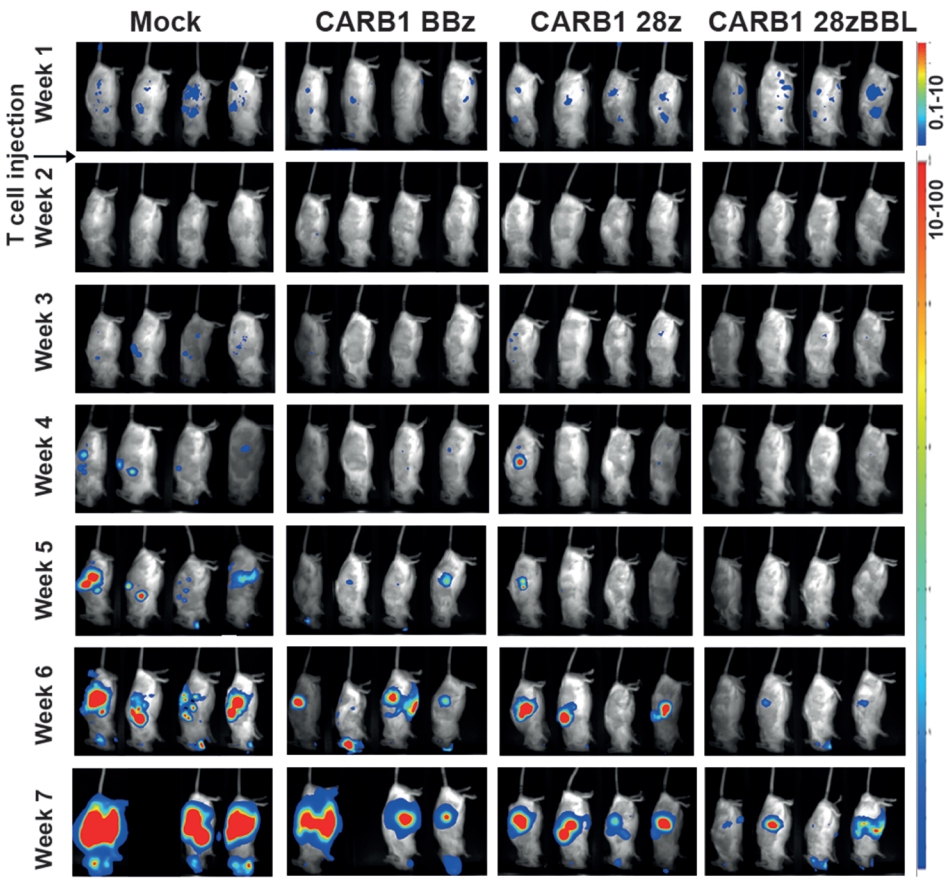
Supplementary Figure 1. CD38 expression on 3T3-CD38. Representative flow cytometry histogram of human CD38 expression on NIH-3T3 wild-type, CD38-transduced NIH-3T3 cells and human MM cell line UM9.



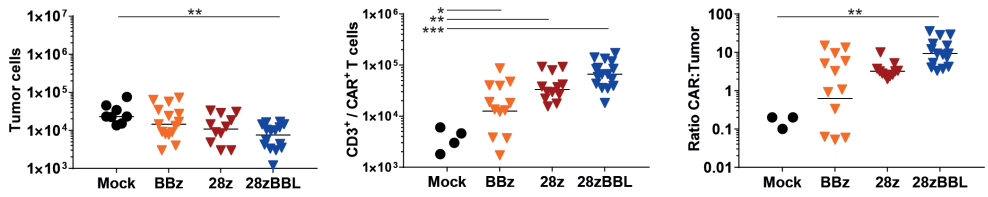
Supplementary Figure 2. Absence of checkpoint markers per costimulation. The percentage of cells expressing no PD-1, Lag3 or TIM3, of all CARs combined per either costimulation BBz, 28z and 28z-BBL. Week 0 is within 1 week after transduction and week 2, after 2 weeks of culturing on 3T3-CD38 cells. Data respresents n=3 donors, mean +/- SEM, * indicates p value <0.05 and ** <0.01 using standard student's t-test analysis.



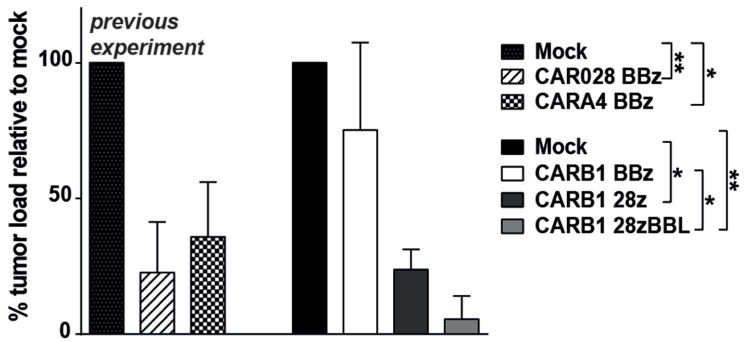
Supplementary Figure 3. Expression of phosphorylated signaling proteins upon exposure to target cells. (A) representative flow cytometry plot of Mock and CARB1-BBz with LNGFR expression and pZap70 or pErk expression. (B) 0, 4, 24 or 144 hours after co-incubation with $CD38^{+}$ target cell line UM9, E:T ratio 1:1. Cells were stained intracellularly for pZap70 or pErk expression, gated on the LNGFR , dsRed or 4-1BBL positive cells. Expression was quantified for the mean fluorescent intensity (MFI) of the CAR+ fraction.



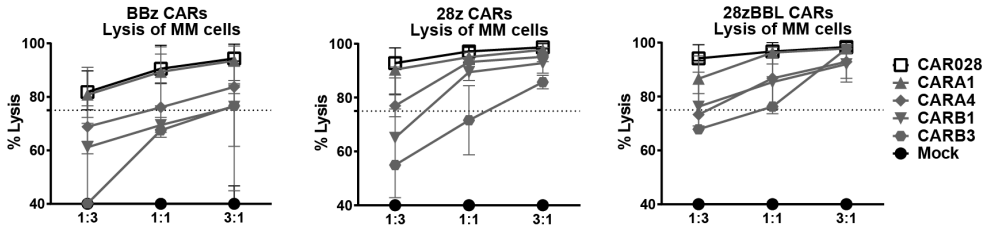
Supplementary Figure 4. Very low-affinity CD38-CAR T cells with 28z costimulation show better tumor control *in vivo*. Mice were i.v. injected with 10 x 10^6 cells of tumor cell line UM9 and treated one week after with i.v. injections of 5 x 10^6 mock, CARB1-BBz, CARB1-28z or CARB1-28zBBL T cells. Week 1-3 depicts representative image of 8 mice per group, week 4-7 consists of 4 mice per group.



Supplementary Figure 5. Post-mortem analysis of scaffolds harboring UM9 tumor cells and infiltrated T cells at week 3. Cells were obtained from scaffolds from sacrificed mice at week 3 of anti-tumor *in vivo* experiment, N=4 per group. Single cells were stained for human mouse and human CD45, counted and analyzed for the % of MM cells measured by flow cytometry. Multiple Myeloma cells ($GFP^+/CD138^+$). Mock or CAR T cells ($CD45^+/CD3^+$ and $LNGFR^+$ or $dsRed^+$ or $4\text{-}1BBL^+$) (Mock $<10^3$ T cells) and the ratio between CAR T cells and MM cells. N=4, results are median cell number of 16 scaffolds (4 per mouse) +/- range. Closed circles (●) indicate mock and open triangles (▽) indicate CD38-CARB1 BBz, closed triangles (▼) -28z and grey closed triangles (▼) -28z-BBL. Indicated are the median values of cell numbers and ratios per group, * indicates p value <0.05, ** <0.01, ***<0.001 using Kruskal-Wallis analysis of variance.



Supplementary Figure 6. 28zBBL costimulation potentiates tumor surveillance of lower affinity CARs *in vivo*. Analysis of tumor load (quantified BLI measurements) at week 7, relative to mock (set at 100%). Results are median of relative tumor load of 4 mice +/- range, * indicates p value <0.05, and ** <0.01 using Mann-Whitney comparison of medians.



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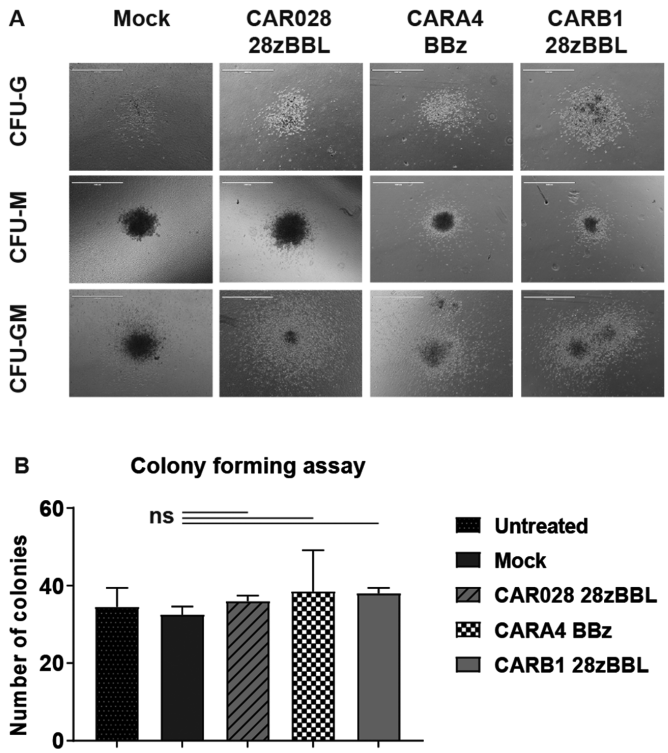
Supplementary Figure 7. Lytic capacity of lower affinity CD38-CAR T cells with different costimulation towards MM in the bone marrow. Bone marrow derived mononuclear cells (BM-MNCs) were co-incubated, mock or CD38-CAR028, A1, A4, B1 or B3 T cells for 16 h. The graphs depict the resulting lysis of $CD138^{+}/CD38^{+}$ cells (MM) , combined data from 4 independent experiments with different patient samples in E:T ratio 1:3, 1:1 and 3:1. The % lysis in these flow cytometry assays was calculated as described in the methods section. N=4, median +/- range.



Supplementary Figure 8. High and low affinity CD38-CAR T cells regardless of their costimulation do not interfere with hematopoietic colony formation. $CD34^{+}$ fraction was isolated from leukapheresis material of a MM patient with easysep positive selection. $CD34^{+}$ cells were co-incubated with mock, CAR028, CARA4 T cells for 4 h at different T:CD34 cell ratio 1:1 before transferring into the semisolid methocult culture medium. After 21 days of culture in plastic dishes, (A) colony-forming unit-monocytes (CFU-M), CFU-granulocytes (CFU-G) and CFU-granulocyte-macrophage progenitors (CFU-GM) were visible, (B) the number of colonies were determined microscopically. Results of a representative experiment are shown mean + SD of duplicates. Colony formation was not significantly (ns) different in a student's t-test.

CHAPTER 5

FEASIBILITY OF CONTROLLING CD38-CAR T CELL ACTIVITY WITH A TET-ON INDUCIBLE CAR DESIGN

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ABSTRACT

Recent clinical advances with chimeric antigen receptor (CAR) T cells have led to the accelerated clinical approval of CD19-CARs to treat acute lymphoblastic leukemia. The CAR T cell therapy is nevertheless associated with toxicities, especially if the CARs are not entirely tumor-specific. Therefore, strategies for controlling the CAR T cell activity are required to improve their safety profile. Here, by using the multiple myeloma (MM)-associated CD38 molecule as target molecule, we tested the feasibility and utility of a doxycycline (DOX) inducible Tet-on CD38-CAR design to control the off-tumor toxicities of CAR T cells. Using CARs with high affinity to CD38, we demonstrate that this strategy allows the proper induction of CD38-CARs and CAR-mediated T cell cytotoxicity in a DOX-dose dependent manner. Especially when the DOX dose was limited to 10 ng/ml, its removal resulted in a relatively rapid decay of CAR- related off-tumor effects within 24 hours, indicating the active controllability of undesired CAR activity. This Tet-on CAR design also allowed us to induce the maximal anti-MM cytotoxic activity of affinity-optimized CD38-CAR T cells, which already display a low toxicity profile, hereby adding a second level of safety to these cells. Collectively, these results indicate the possibility to utilize this DOX inducible CAR-design to actively regulate the CAR-mediated activities of therapeutic T cells. We therefore conclude that the Tet-on system may be more advantageous above suicide-genes to control the potential toxicities of CAR T cells without the need to destroy them permanently.

INTRODUCTION

Over the past years, the clinical successes of chimeric antigen receptor (CAR) engineered T (CAR T) cells have evoked a tremendous enthusiasm for this new mode of immunotherapy in the battle against cancer[1–7]. On the other hand, the increasing clinical experience with CAR T cell therapy have made the investigators aware of the possible severe, even fatal toxicities of this powerful approach[4,8–10]. Several currently known toxicities of CAR T cells are associated with their *in vivo* uncontrolled growth and excess cytokine release soon after infusion in the patients, most probably - though not entirely- due to the on-target off-tumor activities of CAR T cells. This is considered an important concern since virtually all CAR T cells developed to date,, including the most successful CD19-CAR T cells, are directed against tumor-associated, but not entirely tumor specific antigens[11,12].

Set out to develop an efficient CAR T cell therapy for multiple myeloma (MM) we also have recently investigated and demonstrated the possibility to target MM cells with CAR T cells directed against the CD38 antigen, which is highly and uniformly expressed on MM cells[13]. Although CD38 is also expressed on normal hematopoietic cells at intermediate levels, we have shown that CD38-CAR T therapy can be very selective for MM cells, using affinity-optimized CD38-CAR T cells [14]. However, if the affinity of the CAR is not carefully optimized, high affinity CD38-CARs, like many others, can readily cause on-target, off-tumor side effects[8,15,16]. Therefore, an active *in vivo* control of CAR T cell activity is also highly desirable for a safer CAR T cell therapy. Towards this goal, the most frequently proposed and applied strategy is to equip therapeutic T cells with the so-called suicide genes. For instance the herpes simplex virus thymidine kinase (HSV-TK), which converts the prodrug ganciclovir (GCV) into a toxic product [17], or the inducible caspase9, which is dimerized by a small molecule to induce apoptosis[18–20]. The therapeutic T cells can also be engineered to aberrantly express surface antigens like CD20 or EGFR[21,22], which enables their specific targeting via antibodies. While the suicide gene approach had been proven effective in experimental and in the clinical settings, it may not be the ideal strategy to control CAR T cells, since once these genes are activated, the therapeutic effect is also lost permanently. Therefore, actively controlling the CAR expression at the cell surface, rather than killing the CAR expressing T cells, may provide better opportunities to improve their safety profile. Aiming at this goal, several innovative strategies have already been proposed, such as the inducible dimerization of the intra and extracellular domains of the CAR[23–25], or a CTLA-4 signaling-mediated shuttling to the cell membrane[26] (different approaches reviewed in[27] and[28]). While such novel strategies are in full development, a traditional way of controlling the transgene expression is through a tetracycline or doxycycline (DOX) inducible on- (Tet-on) or off- (Tet-off) switch. Indeed, this strategy has recently been successfully applied for controlling the CD19-CAR expression[29].

Due to its relative convenience, we here investigated the utility of the Tet-on inducible CAR design to effectively and timely control the cytotoxic activity of CD38-CAR T cells. Our results demonstrate that the Tet-on CAR design can indeed control the expression of

even high affinity CD38-CARs to effectively allow their CD38-dependent cytotoxic activity in a DOX-dose-dependent manner. Using a carefully defined dose of DOX the CD38-CAR expression and thereby all associated effector functions decayed rapidly upon DOX removal, whereby minimizing the undesired off-tumor effects. Our results thus indicate the feasibility of actively and timely controlling the CD38-CAR T cell activity in case of undesired toxicity associated with on-target, off-tumor effects.

METHODS

Retroviral vector construction

The Tet-on 3G inducible system (Clontech) consists of, the pRetroX-TRE3G vector with the P_{TRE3GV} inducible promoter and the Mock or CAR together with the pRetroX-TET3G for the transactivator protein. The high affinity CD38-CAR028 and low affinities CD38-CARB1 and CARA4 genes were amplified with primers containing the SgrAI and ClaI restriction sites, (forward 5'GGTCCAATCGATATGGCGCTGCCTGTGAGCTC -3', reverse 5'- CGTTACTAGTGGA CACCGG CGTCCTCATCTAG -3'). PCR products were purified using gel-clean up (Bioké) and were subsequently ligated into the pRetroX-TRE3G vector with a T4 ligase (Sigma).

Generation of retroviral particles and transduction of T cells

GP2 293 packaging cells (Clontech) were calcium phosphate transfected with 10 µg pRetroX-TRE-CAR or Mock and pRetroX-TET3G constructs + 5 µg gag-pol (pHIT60), and 5 µg envelope (pCOLT-GALV) vectors (Roche). 16 hours post-transfection complete medium (DMEM + 10% Tet-approved FBS (Clontech) was refreshed, and two and three days after transfection, cell free supernatants containing retroviral particles were collected and directly used for transduction.

Peripheral blood mononuclear cells (PBMCs) from healthy donors were stimulated with lectin-like phytohemagglutinin (PHA-L) (Sigma) in a 6 well plate in culture medium (RPMI-1640, 10% Tet-approved FBS, penicillin; 100 U/ml, streptomycin; 100 µg/ml). After 48 hours, cells were transferred to retronectin (Takara) coated 6-well plates (Falcon). Retroviral transduction was performed by addition of 1 ml TRE-CAR/Mock virus + 1 ml TET virus per well followed by spinoculation (3000 rpm, 1 hour at room temperature) in the presence of 4 µg/ml Polybrene. A second transduction was conducted after 16 hours.6-8 hours after the second hit, half of the cell supernatant was replaced by fresh culture RPMI-1640 + 10% tet-approved FBS + 50 IE/ml rhIL-2 (Proleukin®, Novartis).

Transduced T cell selection and expansion

T cells were selected with neomycine (80 ug/ml) for 1 week and puromycine (5 ng/ml) for 3 days after transduction. Selected T cells were expanded in PMI-1640 (Invitrogen) + 10% Tet-approved FBS (Clontech) + antibiotics (penicillin; 100 U/ml, streptomycin; 100 µg/ml)

using a feeder cell/cytokine mixture consisting of irradiated EBV cell lines of 2 donors (50 Gy) and allogeneic PBMCs of 3 donors (25 Gy),100 U/ml IL-2 and 1 ng/ml PHA.

Cell lines

Unmodified or luciferase (Luc-GFP)-transduced human MM cell lines, UM9 [30] and RPMI8226 [31] were cultured in RPMI-1640 (Invitrogen) + 10% FBS (Invitrogen) + antibiotics (penicillin;100 U/ml, streptomycin; 100 µg/ml) as described [13,14].

Primary cells from MM patients and healthy individuals.

Bone marrow mononuclear cells (BMMC) containing ~20% malignant plasma cells were isolated from bone marrow aspirates of MM patients through Ficoll-Paque density centrifugation and either used directly or cryopreserved in liquid nitrogen until use. PBMCs/MNCs were isolated from Buffy coats of healthy blood-bank donors by Ficoll-Paque density centrifugation. All primary samples were obtained after informed consent and approval by the institutional medical ethical committee.

Soluble CD38 extracellular (sCD38) domain production and staining

Cloning, expression and purification of recombinant CD38 protein was executed as previously described [14]. In brief, cell were washed twice in PBS + 4% human serum albumin, followed by the first staining with sCD38 (30 minutes), washed twice and stained with a PE-conjugated anti-His antibody (Biolegend) for 15 minutes.

Flow cytometry

Flow cytometry assays were performed on BD LSRFortessa. Viable cells were determined with live/dead cell marker (LIVE/DEAD® Fixable Near-IR; Life Technologies L10119). Transduction efficiency and associated CAR expression was measured with an monoclonal antibody towards NGFR-APC (CD271) (clone ME20.4 Biolegend). Monoclonal antibodies used for cytotoxicity assays: CD3-Fitc (clone SK7), CD14-PerCP (clone MoP9), CD19-PerCP (clone SJ25C1) and CD38-PE (clone HB7) (BD Bioscience). CD56-PC7 (clone N901) and CD138-APC (clone BA38) (Beckman Coulter). To distinguish Mock/CAR T cells from target cells, target cell were stained with 0.5 µM Violet tracer (Thermo Fisher C34571) for 25 minutes and washed before cytotoxicity assay co-cultures. Flow cytometry data analysis was performed with FACS Diva 6.1 software.

Cytokine measurements

To determine cytokine production by TRE-Mock and TRE-CAR T cells, cell supernatants were harvested 24 hours after co-culture with MM-BM E:T ratio 3:1. To measure cytokines we used Cytokine Bead Array (CBA) Human Th1/Th2/Th17 cytokine kit (BD) according to manufacturer protocol. In brief, a mixture of capture beads (IL-2, TNF and IFN-γ), PE-

detection reagent and cell supernatant were incubated for 3 hours. Beads were washed and analyzed by a BD standardized flow cytometry assay.

Bioluminescent and flow cytometry-based cytotoxicity assays

One to three days after transduction, selection and expansion, inducible CD38-CAR T cells were incubated with Luc-GFP-transduced human malignant cell lines or violet tracer (Thermo Fisher) labeled primary BM-MNC for 24 hours. The luciferase signal produced by surviving malignant cell lines was determined after 24 hours with a Tecan (Life Sciences) or GloMax® (Promega) 96 Microplate Luminometer within 15 minutes after the addition of 125 µg/mL beetle luciferin (Promega). % lysis cells = (1 − (BLI signal in treated wells / BLI signal in untreated wells)) × 100%. To analyze surviving primary BM-MNCs Flow-Count™ Fluorospheres (Beckman 7547053) were added, cells were harvested and stained for different CD markers (see above). Viable cells were then quantitatively analyzed through Flow-Count-equalized measurements. Percentage cell lysis was calculated as % lysis cells = (1 − (absolute number of viable target cells in treated wells / absolute number of viable target cells in untreated wells)) × 100%.

Statistical analysis

Statistical analyses were performed using Graphpad Prism software version 7.0. For normal distributions parametric student's t-tests were used. In analyses where multiple groups were compared, either a parametric ANOVA with bonferroni posthoc test or nonparametric Kruskal-Wallis test were used with subsequent multiple comparison. A p value <0.05 was considered significant.

RESULTS

DOX dependent induction of CD38-CAR expression

To evaluate the controllability of CAR expression with an inducible design, we first generated a Tet-on inducible second generation CD38-CAR, which contained single chain variable fragments (scFv) with a high CD38 affinity, including the 4-1BB and CD3z signaling domains. This CAR gene was put under the regulation of a third generation pTre, containing seven tetracycline responses elements (TRE) followed by a minimal CMV promotor (Fig. 1). The construct also contained the low affinity nerve growth factor receptor (LNGFR), separated from the CAR gene by a P2A sequence. The control mock vector contained only the LNGFR marker gene (Fig. 1A). Upon retroviral transduction with this inducible construct (TRE-CD38-CARs), the T cells showed no detectable CAR expression in the absence of DOX (Fig. 1A) but expressed high levels of the CAR within 48 hours of exposure to a high dose[32] of DOX (Fig. 1B). All transduced cells, including the mock-transduced cells also expressed the LNGFR marker gene (Fig. 1B). We then determined the cytotoxic activity of T cells transduced with this inducible CD38-CAR against two $CD38^+$ MM cell lines UM9 and RPMI8226 with or without pre-treatment with DOX. As expected, there was no CAR

expression and no CAR-mediated lysis in the absence of DOX. In contrast, DOX- treatment induced CAR expression and resulted in the effective lysis of both MM cell lines (Fig. 2A).

DOX dose-dependency of CD38-CAR expression and cytotoxic activity

We next evaluated whether the level of CD38-CAR expression and the CD38-dependent cytotoxic activity of TRE-CD38-CAR T cells could be regulated by the dose of DOX, by incubation with serial concentrations of DOX ranging from 1-1000 ng/ml for 48 hours (Fig. 2B)[32,33]. The CAR expression was maximal at 1000 ng/ml of DOX, but gradually decreased by lowering the dose, which was also reflected in the cytokine production (supplemental figure S1). A 5-fold lower expression level was reached at a DOX dose of 10 ng/ml (Fig. 2B). The cells showed a very low CAR expression at a dose 1 ng/ml, which

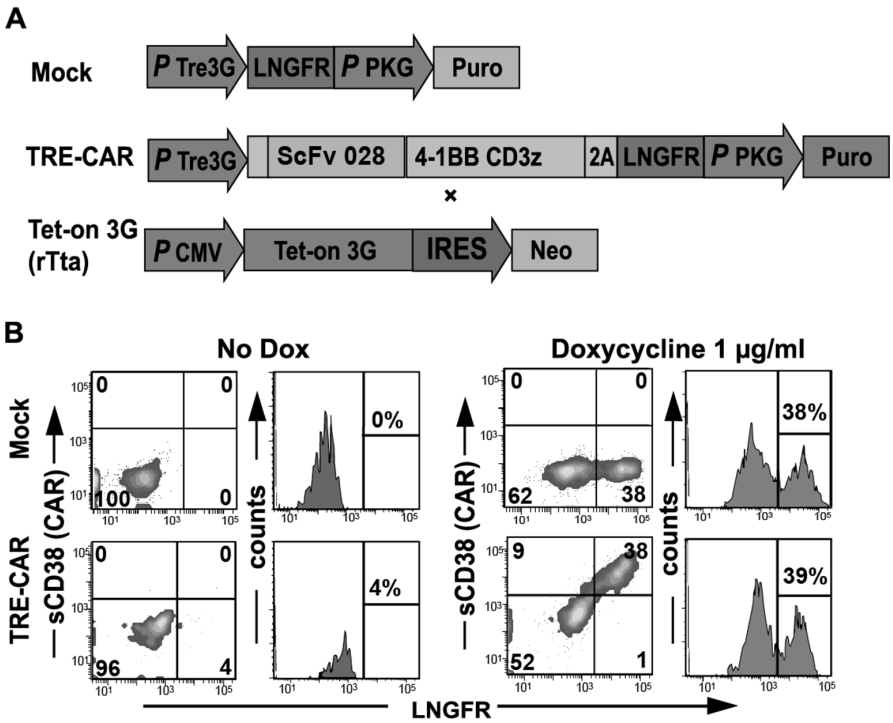

Figure 1. DOX induced CD38-CAR expression (A) Schematic overview of constructs. The pRetroX-TRE3G vector with the P_{TRE3GV} inducible promoter controlling the transcription of Mock containing the marker LNGFR or the CD38-CAR (high affinity scFv 028 or low affinities A4 or B1), consisting of the single chain variable fragments, 4-1BB and CD3ζ and a LNGFR separated by a P2A sequence. These vectors were co-transduced with the pRetroX-TET-On 3G containing the transcription site for the transactivator protein rTta. (B) Flow cytometry density plots and histograms to determine CAR expression of the inducible CAR T cells, after 48 hours incubation with 0 or 1000 ng/ml DOX. The expression of the marker LNGFR was measured with an APC-conjugated antibody. CAR expression was measured by binding of his-tagged (HHHHHH) soluble CD38 (sCD38) protein to the ScFv domain, stained with PE-conjugated anti-His tag antibody.

was not distinguishable from DOX untreated conditions. The cytotoxic activity of the T cells also significantly and proportionally decreased by lowering the dose of DOX, from 95% of lysis at 1000 ng/ml to 63% of lysis at 10 ng/ml DOX at an effector: target ratio of 10:1 (Fig. 2C). Again here, no CD38-mediated cytotoxic activity above non-specific (mock) levels was observed from DOX untreated TRE-CD38-CAR T cells, indicating that there was no functional "leakage" in this inducible system, despite the fact that a very low level of CD38-CAR was detectable on the cell surface (Fig. 2B).

Decay kinetics of CAR expression and CD38-dependent on-tumor cytotoxic activity of TRE-CD38-CAR T cells after DOX removal

After showing the DOX dose-dependency of CAR expression, we studied the induction and decay kinetics of the CD38-CARs upon exposure and after the withdrawal of serial concentrations of DOX (supplemental figure S2). We also evaluated the CD38-dependent cytotoxic activity of the cells against MM cells in these assays. The experimental set-up

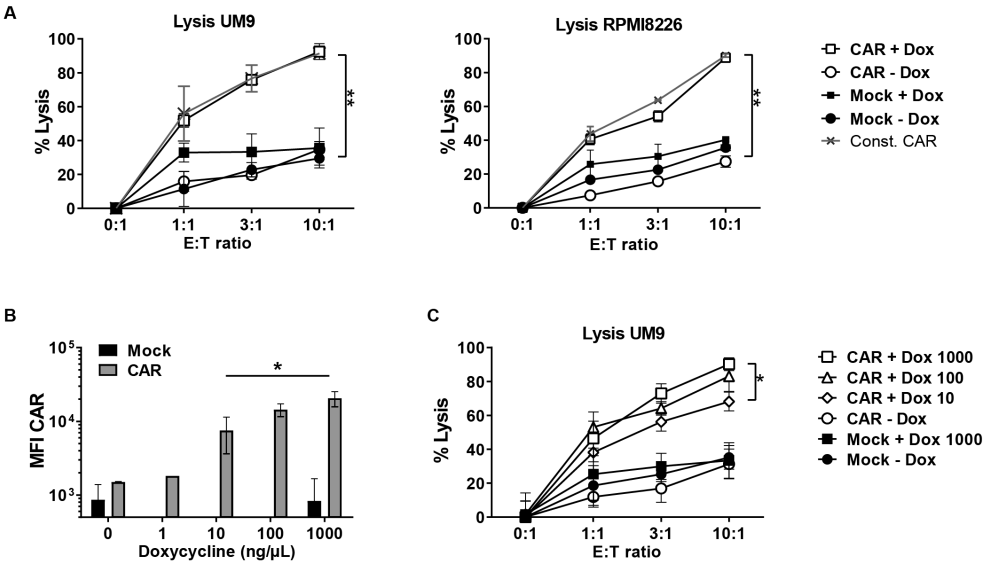

Figure 2. DOX dose-dependent induction of CD38-CAR expression and anti-MM cytotoxicity. (A) Lysis of luciferase-transduced CD38$^+$ MM cell lines UM9 and RPMI8226 after co-incubation with Mock and inducible (high affinity 028) CD38-CAR, which were treated with no or 1000 ng/ml DOX for 48 hours. (grey lines indicate lysis by constitutively (const.) expressed high affinity 028 CD38-CAR T cells) The BLI signal from surviving MM cells was measured after 16 hours using a luminometer and the percentage lysis was calculated as indicated in the material & methods. n=2 for each condition mean+/- SD (B) Mean fluorescent intensity (MFI) of the CAR measured by staining with soluble CD38-his after 48 hours incubation with 0, 1, 10, 100 or 1000 ng/ml DOX, n=2 +/- SD. (C) The cytotoxic activity of untreated or DOX treated inducible CD38-CAR T cells against luc+ MM cell line UM9 after 16 hours. Similar results were obtained in independent assays, n=2 +/- SD. * indicates p value <0.05 and ** <0.01 using one-way analysis of variance and subsequent multiple comparison.

is depicted in figure 3A and time and concentration-dependent CAR expression levels in Fig. 3B. Six hours of DOX treatment induced a slight to moderate CAR expression, even with 1000 ng/ml of DOX. This moderate expression was retained until 48 hours after DOX removal by extensive washing of the cells (Fig. 3C left panel). These CAR expression levels resulted, after washing off DOX, a maximal 30% CD38-CAR-mediated lysis of MM cells above mock control (Fig. 3D left panel), which showed a delayed increase in cytotoxicity, possibly due to the relatively slow transcription induction and subsequent functionality.

A 24-hour DOX-treatment resulted in substantial CAR expression (Fig. 3C middle panel) and effective CD38-CAR mediated lysis of MM cells (Fig. 3D middle panel). The CAR decay upon DOX removal occurred in a linear fashion, with a slightly faster decay kinetics for the cells incubated with 10 ng/ml of DOX as compared to 1000 ng/ml. The cells expressed very low but detectable levels of CARs 24 hours after DOX removal, but no CAR expression was detectable 48 hours after DOX removal (Fig. 3 C middle panel). The cytotoxic activity of the cells decreased in a significantly faster kinetics after 10 ng/ml DOX incubation. No significant CAR mediated lysis of MM cells above mock control could be observed 24h after of removal of 10 ng/ml DOX (Fig. 3D, middle panel).

As expected, the CAR expression was highest after 48-hour DOX treatment. The CAR decay kinetics was similar to 24-hour incubated cells but the cells still expressed intermediate to moderate levels of CAR for longer periods, since the initial levels were higher (Fig. 3 C right panel). Consequently, the cells treated with 1000 ng/ml DOX did not significantly downregulate their high anti-MM activity, while a 30-40% CAR mediated cytotoxic activity against MM cells remained even 48-120 hours after the removal of 10 ng/ml DOX (Fig. 3D right panel). When we correlated the CAR expression levels and the lysis levels in these assays, we observed a strong correlation between CAR expression and CAR-mediated lysis of the MM cells for both 1000 (Fig. 3E top panel) and 10 ng/ml of DOX (Fig. 3E lower panel).

Decay kinetics of CD38-dependent off-tumor cytotoxic activity of TRE-CD38-CAR T cells after DOX removal

An important aim of the inducible CAR-design is to effectively and rapidly control the on-target off-tumor mediated toxicities of CD38-CAR T cells. Therefore, in the further evaluation of TRE-CD38-CAR T cells we not only tested their anti-MM activity but also evaluated the potential off-tumor toxicities against $CD38^{int}$ normal hematopoietic cells upon exposure and after removal of DOX. To test this in a most relevant way, we used BM samples from MM patients, which contain not only $CD38^{hi}$ MM cells but also $CD38^{int}$ normal hematopoietic cells as target cell populations. After the induction of CAR expression the TRE-CD38-CAR T cells were incubated with BM-MNC and the CAR-dependent lysis of various cell subsets was determined by flow cytometry-mediated assays as described previously [13,14]. An illustrative example of such an assay is depicted in figure 4A. In figure 4B, both 24h and 48h stimulation with 10 or 1000 ng/ml DOX of TRE-CD38-CAR T cells

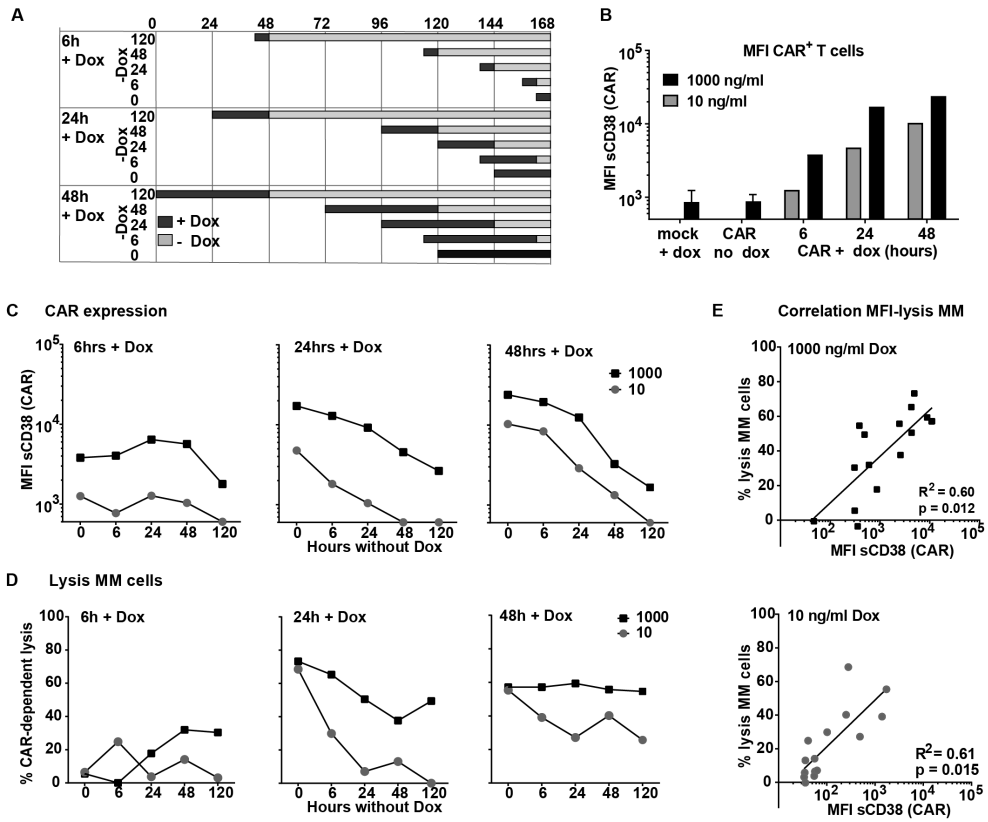

Figure 3. Induction and decay kinetics of CD38-CAR expression. (A) schematic overview of CAR induction and decay assay. Black bars indicate the DOX incubation times, gray bars indicate the period of decay after the removal of DOX. (B and C) Representative results (in total of n=3 repeated measurements) of mean fluorescent intensity (MFI) of the CAR measured by staining with soluble his-tagged CD38 after 6, 24 or 48 hours incubation with (B) 10 or 1000 ng/ml DOX or 6, 24, 48 or 120 hours after washing of DOX (C) (an MFI of 600 was considered background expression). (D) A MM patient bone marrow sample with 20% MM cells was co-incubated with inducible (high affinity 028) CD38-CAR T cells (E:T ratio 3:1) treated with DOX according to the schedule figure 3 A. Depicted are the CAR-dependent % of lysed MM cells ($CD138^+/CD38^+$) (% lysed by CAR - % lysed by Mock). (E) Significant pearson correlation of MFI of CAR expression as detected with soluble CD38 (sCD38) with % lysis of MM cells. High dose DOX R^2=0.60 and p=0.012, low dose DOX R^2=0.61 and p=0.015.

resulted in substantial CAR-mediated of MM cells. As expected from the high affinity CD38-CAR T cells there was also considerable lysis of $CD38^+$ non-MM cells in the bone marrow. After the removal of DOX, however, the TRE-CD38-CAR T cells which were treated with 10 ng/ml DOX rapidly lost their off-tumor effects within 24 hours, while there was still 50%-MM activity left especially of CAR T cells that were exposed to DOX for 48 hours. 120 hour after DOX removal only the cells that were exposed to 1000 ng/ml DOX retained some anti–MM activity; all off-tumor activity was lost. These results indicated that the off-tumor activities of high-affinity CD38-CAR T cells can be readily and rapidly down-

regulated after exposure of the cells even 48 hours to relatively low doses of (10 ng/ml) DOX, while some anti-MM reactivity still retained.

Low-affinity inducible CD38-CAR T cells

The experiments addressing the on- and off-tumor effects of inducible CD38-CAR T cells indicated that gradual decay of CAR expression after DOX removal not only allows the rapid and effective control of the off-tumor toxic effects of the high affinity CD38-CAR T cells but may also generate a small, albeit a temporary therapeutic window in which the on-tumor effects can be maintained. Thus not only optimally lowering the affinity of CD38-CARs, as we have recently shown[14], but also optimally lowering the CAR expression on the cell surface seemed to result in discrimination of $CD38^{high}$ MM cells from $CD38^{int}$ normal hematopoietic cells. Therefore we finally questioned whether these two strategies can be combined to make much safer CAR T cells. Hence we generated inducible CD38-CAR T cells from (two) a low affinity CARs (CAR A4 and B1), which already displayed much less off-tumor toxicity profiles than the high affinity CD38-CAR T cells (Fig. 5 and supplemental

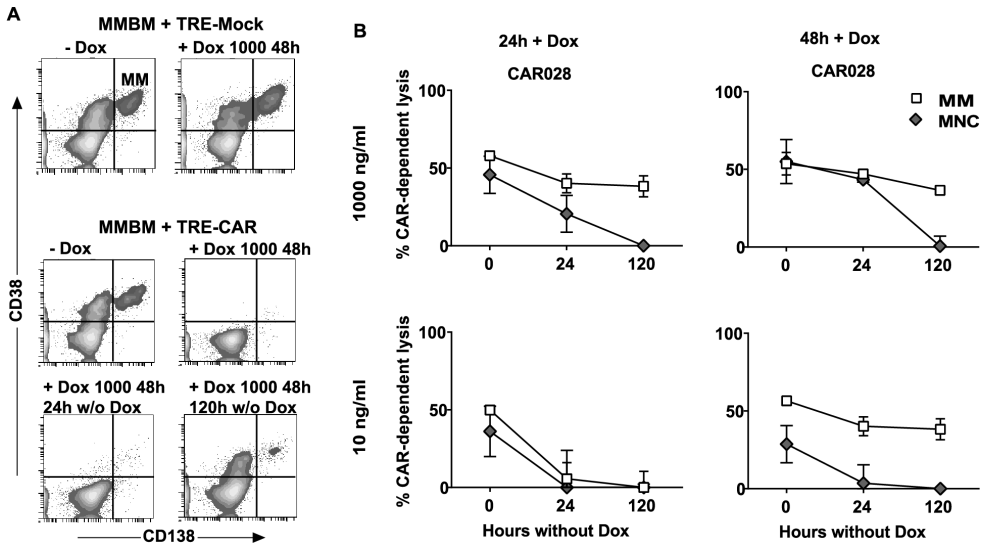

Figure 4. Off-tumor effect of inducible CD38-CAR T cells. (A) Representative flow cytometry density plots of MM-BM with $CD38^{+}/CD138^{+}$ cells (MM) after treatment with inducible mock (+/- 1000 ng/ml DOX for 48 hours) and inducible high affinity (028) CD38-CAR T cells (- DOX or + 1000 ng/ml DOX for 48 hours and 0, 24 or 120 hours after DOX removal). (B) MM patient bone marrow samples (n=3 different patients A, B, C, illustrated in supplemental figure S3) with ~20% MM cells were co-incubated with inducible (high affinity 028) CD38-CAR T cells (E:T ratio 3:1) treated with DOX according to the schedule figure 3 A. Depicted the mean CAR-dependent % lysis of MM ($CD138^{+}/CD38^{+}$; open squares) and % lysis of healthy non-MM cells ($CD138^{-}/CD56^{-}/CD38^{+/-}$; grey diamonds) by inducible CD38-CAR T cells. Incubated with DOX for 24 hours 1000 ng/ml (upper left), 48 hours 1000 ng/ml (upper right), 24 hours 10 ng/ml (lower left), 48 hours 10 ng/ml (lower right). Mean values of n=3 patients +/- SEM.

figure S4). After induction of CAR expression with 10 or 1000 ng/ml DOX the CAR T cells displayed substantial CAR-mediated lysis against primary MM cells in the BM-MNC, but, as expected, there was little or no lysis of $CD38^+$ non-MM cells even after using 1000 ng/ml of DOX. More interestingly, the cells maintained their lytic activity against MM cells, with no signs of off-tumor effects for longer than 120 hours after the removal of DOX (Fig. 5). Taken together these results demonstrated that the DOX inducible CAR design is also a feasible strategy for low-affinity CD38-CAR T cells to establish their therapeutic effects, which could be maintained with no toxic signs for a longer period even after removal of DOX.

DISCUSSION

In this study we evaluated the feasibility of controlling the on-target, off- tumor effects of CD38-CAR T cells using a DOX inducible Tet-on CAR design. Our results show that this inducible strategy allows the sufficient surface expression of CD38-CARs and thus also the CAR-mediated cytolysis of relevant target cells in a DOX-dose dependent manner. More importantly, we found that the removal of DOX results in a gradual elimination of

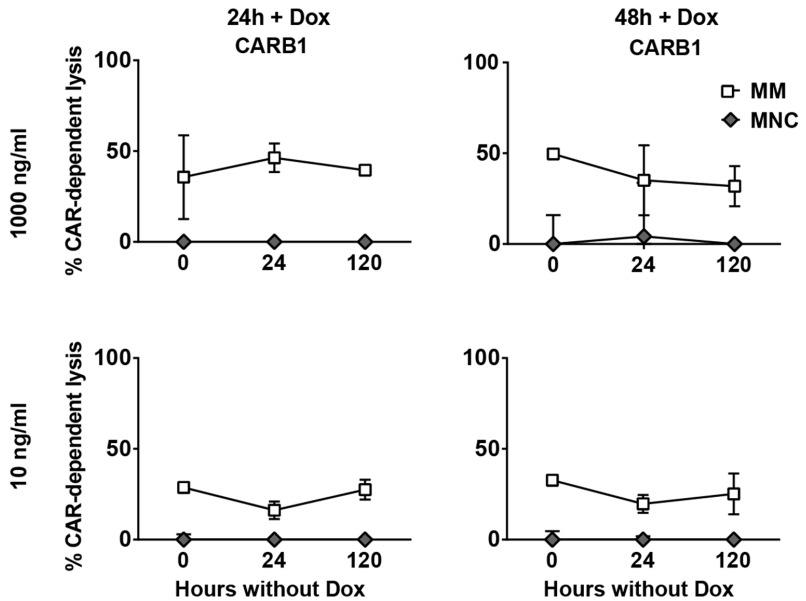

Figure 5. Off-tumor effect of inducible low affinity CD38-CAR T cells. MM patient bone marrow samples (n=2) with ~20% MM cells were co-incubated with inducible low affinity (B1) CD38-CAR T cells (E:T ratio 3:1) treated with DOX according to the schedule figure 3A. Depicted are the average CAR-dependent lysis of MM cells ($CD138^+/CD38^+$; open squares) and lysis of healthy non-MM cells ($CD138^-/CD56^-/CD38^{+/-}$; grey diamonds) by inducible CD38-CAR T cells. Incubated with DOX for 24 hours 1000 ng/ml (upper left), 48 hours 1000 ng/ml (upper right), 24 hours 10 ng/ml (lower left), 48 hours 10 ng/ml (lower right). Mean values n=2 patients (B and C, same patients Fig 4, indicated in supplemental figure S3.) +/- SEM.

the off-tumor hematotoxic effects of the high affinity CD38-CAR T cells, especially when DOX exposure dose was limited to 10ng/ml. Somewhat unexpectedly we observed that this approach also generated a small, albeit a temporary therapeutic window after DOX removal, in which the on-tumor effects retained much longer and therefore could be separated from the off-tumor effects, which may be explained by higher CD38 levels on MM cells as compared to normal hematopoietic cells. We also collected evidence that this inducible strategy can also be applied to induce the maximal functional expression of our recently developed affinity-optimized CD38-CARs, which already enable T cells to discriminate $CD38^{high}$ MM cells from $CD38^{int}$ normal hematopoietic cells. This will provide another level of safety to already affinity optimized CD38-CAR T cells. Collectively, these results indicate the possibility to utilize this DOX inducible CAR-design to actively regulate the CAR-mediated activities of therapeutic T cells.

From a cell biological point of view, our data collected during the induction and decay phase of high affinity CD38-CARs provide perhaps for the first time a direct evidence for the linear correlation between the CAR surface expression levels and the cytotoxic activity of CD38-CAR T cells. Related to this, our finding that a certain CAR expression level results in the separation of the anti-tumor effects of CD38-CAR T cells from their off-tumor cytotoxicity is also novel and suggest that quantitative manipulation of CAR surface expression could, next to CAR affinity optimization, also be exploited to increase the tumor selectivity of CARs directed against tumor-associated antigens (TAAs). Nonetheless, it should be noted that the regulation of gene expression is a highly complex and a difficult to control process. Although we provide evidence that CAR expression in this inducible system is dependent on the dose of DOX, a phase 1 gradual DOX increase may be needed to define a precise dose for each individual to establish a certain level of functional CAR expression. Thus whether the DOX inducible Tet-on strategy will be feasible and the most convenient way for quantitative control of CAR surface expression needs to be evaluated in future studies. Alternative strategies which are not based on gene regulation may be more feasible for the precise quantitative expression of CARs on the T cell surface, such as the docking of an scFv on an universal receptor[25].

From the safety point of view, our data clearly indicate that the removal of DOX will eventually result in the elimination of off-tumor toxicities of CD38-CAR T cells. However, one important issue to be discussed is whether the elimination of toxic effects will be sufficiently rapid with this DOX-inducible system. We observed that high affinity CD38-CARs, when they are induced with an optimal dose of 10ng/ml of DOX will lose most, if not all, of their off-tumor effects within 24 hours (Fig. 4). This time frame, when compared to the reported much more rapid clinical results of the suicide gene inducible caspase 9 (iCasp9) system (90% of the cell kill within 30 minutes) is indeed too slow[19]. However, in an *in vitro* system the elimination of iCasp9 positive T cells took also around 24 hours[20]. Moreover, it is known that suicide gene approaches, for instance HSV-TK can cause bystander effects and thereby exhibit safety "leaks"[34,35]. Furthermore, it needs to be mentioned that it is not precisely known how rapid the elimination of the infused cells

should be to prevent further complications. If the direct hematotoxic effects of CD38-CAR T cells needs to be controlled, this time frame does not necessarily be very rapid, since CD38-CAR therapy mainly eliminates NK cells and monocytes, while a large fraction of T and B cells will be ignored as they are CD38 low/negative. The temporary damage to NK cells and monocytes can be gradually recovered after DOX removal since CD38-CARs therapy does not affect CD38 negative normal stem cells[13,14].

In the case of CRS associated with CAR therapy, the immediate disease symptoms can be currently controlled within hours with the use of the anti-IL6 antibody tocilizumab[6,10,36]. Thus, this novel treatment generates a window of opportunity, during which the downregulation of CARs to sufficiently non-toxic levels can be realized. In conclusion, although it may be not as rapid as the suicide gene approach, the inducible CAR strategy can be beneficial, especially when used in combination of tocilizumab in case of CRS. Obviously the main advantage of such an approach will be the control of the therapeutic cells without the need to destroy them permanently.

Another relevant question is whether the Tet-on system is the best or the most convenient system among other inducible systems: when compared to a Tet-off system, a Tet-on inducible system seems more practical to control the toxic effects of CAR T cells since a Tet-on system, where the default is the "off" switch will prevent unfavorable antigen-induced T cell differentiation and exhaustion as compared to a default "on" switch[37]. However, several recently developed strategies such as the CARs with a dimerizer-activated signaling domains [23] may be more effective and rapid than the Tet-on system because they are not dependent on transcriptional gene regulation to achieve the similar effect. Strategies aiming at the induction of CARs in the tumor microenvironment, such as hypoxia inducible CARs[38] or the recently described Syn-Notch strategy[39,40] in which the CAR expression is induced upon recognition of an antigen in the microenvironment also deserve comparison with this more conventional Tet-on strategy. Therefore, while our results are highly promising, we need to acknowledge that an *in vivo* preclinical evaluation of this Tet-on system is necessary to determine whether this strategy can indeed control the CAR T cell related toxicities. However, the challenge here is the development of appropriate *in vivo* models where the hematotoxic and other off-tumor effects, as well as the CRS related to CAR T cell therapy, can be adequately mimicked[41].

Nonetheless, the flexibility in CAR functions that we observe in this study illustrates the advantages to control CAR expression and consequently the cytotoxic functions. The development of a controllable switch to effectively tune engineered cells will lead to a safer application of CAR T cell therapy.

ACKNOWLEDGEMENTS

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DISCLOSURES

There is no relevant potential conflict of interest to disclose.

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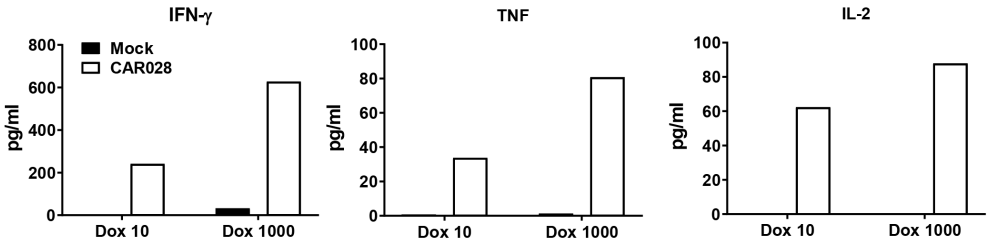
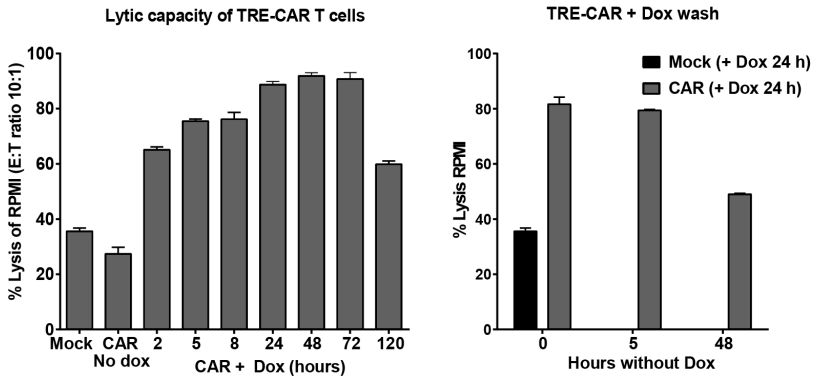
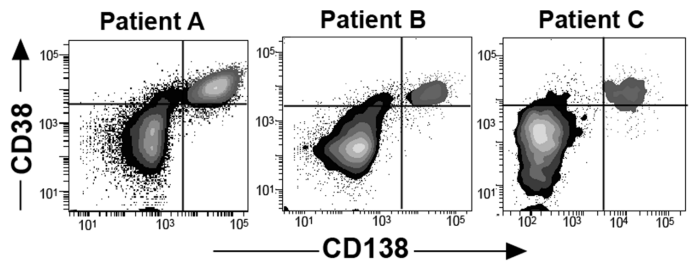
SUPPLEMENTARY MATERIAL



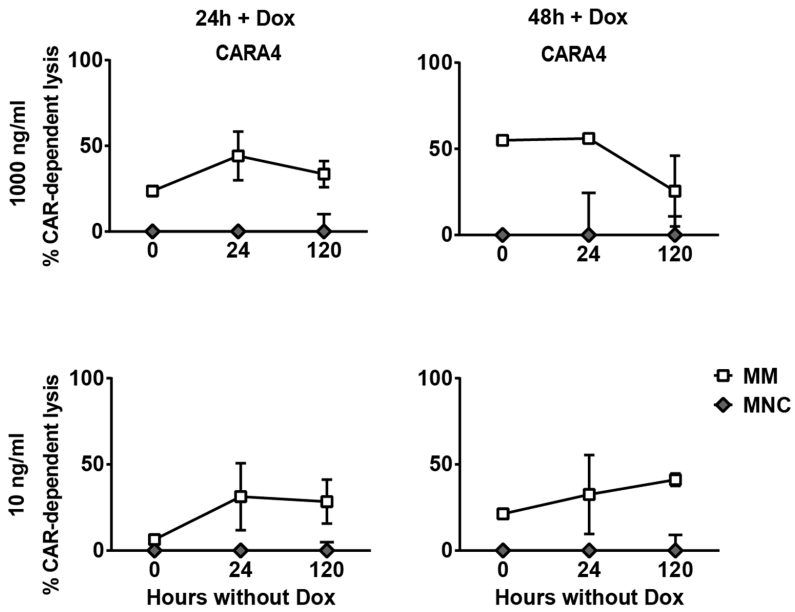
Supplementary Figure 1. TRE-CAR T cells show a DOX-dependent cytokine release upon incubation with MM-BM. 24 hours after co-incubation with MM-BM, cell supernatants were harvested to measure cytokine secretion (E:T ratio 3:1) with a flow cytometry-based assay. Graph shows the secretion of IFN-γ, TNF and IL-2.



Supplementary Figure 2. Different time points for induction of CD38-CAR-induced anti-MM cytotoxicity. Lysis of luciferase-transduced CD38$^+$ MM cell line RPMI8226 after co-incubation with inducible Mock and TRE-CD38-CAR, which were treated with (A) no or 1000 ng/ml DOX for 2, 5, 8, 24, 48, 72 and 120 hours or (B) treated with Dox 24 hours and washed and incubated without DOX for 5 or 48 hours. The BLI signal from surviving MM cells was measured after 16 hours using a the luminometer and the percentage lysis was calculated as indicated in the material & methods.



Supplementary Figure 3. Representative flow cytometry density plots of MM-BM. MM-BM samples of patient A, B and C were stained for CD38$^+$/CD138$^+$ expression to illustrate the level of CD38 expression on MM cells (upper right, in red) versus healthy MNCs (upper and lower left, in purple).



Supplementary Figure 4. Off-tumor effect of inducible low affinity CD38-CARA4 T cells. MM patient bone marrow samples (n=2) with ~20% MM cells were co-incubated with inducible low affinity (A4) CD38-CAR T cells (E:T ratio 3:1) treated with DOX according to the schedule figure 3A. Depicted are the average CAR-dependent lysis of MM cells ($CD138^+/CD38^+$; open squares) and lysis of healthy non-MM cells ($CD138^-/CD56^-/CD38^{+/-}$; grey diamonds) by inducible CD38-CAR T cells. Incubated with DOX for 24 hours 1000 ng/ml (upper left), 48 hours 1000 ng/ml (upper right), 24 hours 10 ng/ml (lower left), 48 hours 10 ng/ml (lower right). Mean values n=2 patients (B and C, same patients Fig 4) +/- SEM.

CHAPTER 6

INKT CELLS AS CARRIERS FOR THERAPEUTIC CD38-CARS TO TARGET MULTIPLE MYELOMA

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Under revision

ABSTRACT

Va24-invariant natural killer T (iNKT) cells can respond to tumor cells presenting certain glycolipids such as α-galactosylceramide in the HLA-like molecule CD1d. Increased numbers of iNKT cells are frequently associated with a decrease in tumor load and improved clinical outcome. The CD1d-restricted reactivity of iNKT cells diminishes their toxicity profile, with a significantly reduced risk for graft-versus-host disease when they are administered across HLA-barriers. To maximally harness the broad applicability of iNKT cells for multiple myeloma (MM) treatment, we now additionally armed them with a CD38-specific chimeric antigen receptor (CAR) which, due to its attenuated affinity, exclusively targets MM cells with high levels of CD38 expression. We demonstrate that CD38-CAR iNKT cells effectively recognize and kill $CD38^{high}$ malignant cell lines, primary MM cells, with an additive cytotoxic effect upon CD1d engagement through their endogenous invariant T cell receptor (TCR). Due to the attenuated CD38-CAR affinity, the cytotoxicity of dual-specific CD38-CAR iNKT cells is largely directed at MM cells, while $CD38^{neg}$ and $CD38^{int}$ healthy hematopoietic cells are ignored. Further indicating their therapeutic utility, CD38-CAR iNKT cells displayed a Th1-cytokine profile and expressed low levels of exhaustion makers. Finally, we demonstrate that CD38-CAR iNKT cells are able to vigorously expand for several weeks in a CD38-dependent manner and can be further expanded without losing CD38-specific functions, with α-galactosylceramide pulsed or unpulsed dendritic cells, suggesting that dendritic cells can be used for *ex vivo* and *in vivo* expansion of iNKT cells for therapeutic applications.

INTRODUCTION

Over the past years, T cells engineered to express chimeric antigen receptors (CAR) have shown great successes in the battle against hematopoietic malignancies. Nonetheless, there are still important hurdles to be taken in order to make CAR therapy more effective, safe and universally accessible. In particular, the necessity to use the patients' autologous polyclonal T cells for the production of CAR T cells seem to generate a considerable variation in the efficacy of the therapy, since the quality of patient T cells can be greatly influenced by previous treatment schemes and/or the tumor type of the patients[1–3]. Hence, a patient-independent, off-the-shelf available CAR therapy is the ultimate goal of many investigators[4–8]. Application of CAR T cells across HLA-barriers however carries the risk of severe Graft versus Host disease (GVHD) due to the expression of potentially allo-reactive endogenous TCRs[9–11]. Therefore, several approaches has been proposed to silence the endogenous TCR expression of CAR T cells with novel gene editing technologies[12–14]. Alternatively, other killer immune cell subsets are currently being explored as "universal" CAR carriers. An appealing candidate is the population consists of NK cells[15–20], as they are highly cytotoxic but not associated with GvHD[21]. Furthermore NK cells display a relatively short life-span, which could limit the potential on-target off-tumor toxicities of CAR directed cellular therapies. On the other hand, building a long term memory may not be possible with NK cells. Alternatively, CARs can be carried by another subset of innate-like, but antigen-specific immune cells which can sustain a long term memory: the invariant natural killer T (iNKT) cells. iNKT cells recognize glycolipid antigens presented by CD1d with their defined and invariant T cell receptor (TCR), consisting in humans of a Vα24 chain paired with a Vβ11 chain[22–24]. Due to this specific antigen-recognition pattern, iNKT cells are not associated with GvHD and might even be protective without hampering the Graft versus Tumor (GvT) effect[25,26]. Furthermore, iNKT cells possess therapeutic potential. Their infiltration in tumor sites has been associated with better disease outcome[27] and reduced iNKT cell numbers and function have been associated with disease progression in multiple myeloma (MM)[28], prostate cancer[29] and several other malignancies[30].

Although, iNKT cells represent a small fraction in the peripheral blood (0.003 – 0.7 % of CD45+ cells)[31], they display strong cytolytic function in a TCR dependent fashion[32], and demonstrate rapid expansion *ex vivo* when stimulated with α-galactosylceramide (α-GalCer) loaded antigen presenting cells[19,31,33]. Also in clinical studies *in vivo* stimulation of iNKT cells with α-GalCer loaded antigen presenting cells enhanced iNKT activity and decreased patient's tumor load[34–36].

Prompted by the therapeutic potential of iNKT cells, the possibility to expand them *ex vivo* and to boost them *in vivo* through triggering their endogenous TCR, we aimed at maximally exploiting iNKT cytotoxic function towards MM, by additionally arming them with affinity attenuated CD38-CARs recently developed in our laboratory. Due to their attenuated affinity, these CD38-CARs exclusively target MM cells with high levels of CD38 expression while they largely ignore healthy hematopoietic cells expressing

normal levels of CD38[37,38]. Since we have recently found that the costimulatory domain of these CARs can influence their cytotoxic as well as proliferative capacity (manuscript submitted for publication). iNKT cells were transduced with either i) a CD28 costimulatory domain, ii) a 4-1BB costimulatory domain or iii) a CD28 costimulatory domain combined with a separately expressed 4-1BBL. The latter enables 4-1BB signaling in an autologous/paracrine fashion[39,40].

After transducing iNKT cells with these CD38-CARs, we demonstrated cytotoxic activity against MM cells via CD38-CARs as well as via the endogenous TCR. We furthermore analyzed therapeutically important parameters, including their potential off-tumor toxicity towards normal hematopoietic cells, their cytokine profile, phenotype and their ability to expand either via stimulation through the CD38-CAR or through the endogenous TCR via stimulation with a-GalCer pulsed dendritic cells.

METHODS

Lower affinity CD38-CARs

CD38-CARs with attenuated affinity were produced with different germline variable light chains while keeping the variable heavy chain of a high affinity antibody (clone 028) constant as previously described[38]. In the retroviral construct, the scFv was followed by a CD8a transmembrane domain and the 4-1BB and CD3ζ signaling domains or a CD28 transmembrane and intracellular sequence as described in Zhao *et al.*[40]. in different constructs, the CAR sequences were linked by a P2A sequence[47] to a truncated LNGFR, dsRed or 4-1BBL sequence, as depicted in figure 1A.

The 4-1BBL sequence was obtained form EBV-LCL cell line 10850, amplified by standard RT-PCR (Thermo Fisher) as described (manuscript submitted).

Magnetic activated cell sorting (MACS) of iNKT cells and DCs

Healthy donor blood PBMCs were isolated from buffy coats through Ficoll-Paque (GE Healthcare Life Sciences) density centrifugation. iNKT cells were isolated from PBMCs using iNKT specific MicroBeads, or CD14 specific microbeads to isolate DCs, following the instructions of the manufacturer (Miltenyi).

Generation of retroviral particles and transduction of iNKT cells

Phoenix-Ampho packaging cells were transfected with 10 μg CAR constructs + 5 μg gag-pol (pHIT60), and 5 μg envelope (pCOLT-GALV) vectors (Roche) using the calcium phosphate method, as described elsewhere. Sixteen hours post-transfection complete medium (DMEM + 10% FBS) was refreshed, and two and three days after transfection, cell free supernatants containing retroviral particles were collected and directly used for transduction of iNKT cells, which were pre-stimulated for 48 hours with a cell irradiated feeder mixture containing PBMCs from 2-3 different healthy donors ($3x10^6$/well), lectin-like phytohemagglutinin (PHA-L; 1mg/ml) in culture medium (RPMI-1640), supplemented with

10% FBS, 100 IU/ml sodium penicillin, 100 µg/ml streptomycin sulphate Transductions were performed in retronectin coated (15 µg/ml) (Takara) 24-well plates (Falcon) at a density of 5-10 x10^5/ml of iNKT cells in 0.5 ml by addition of 0.5 ml virus per well followed by spinoculation (3000 rpm, 1 hour at room temperature) in the presence of 4 µg/ml Polybrene. A second transduction was conducted after 16 hours, replacing 2/3 of the cell supernatant with freshly obtained virus (0.5 ml). 6-8 hours after the second hit, half of the cell supernatant was replaced by fresh culture RPMI-1640 + 10%FBS and 50 IE/ml rhIL-2 (Proleukin®, Novartis) was added once. Seventy-two hours post-transduction LNGFR, dsRed or 4-1BBL and CD38 expression were measured by flow cytometry to determine transduction efficiency.

Primary Bone Marrow MNCs cells from MM patients

Bone marrow mononuclear cells (BM-MNCs) from MM patients were isolated from bone marrow aspirates containing ~10-40% malignant plasma cells (determined by flow cytometry as $CD56^{+/-}$/$CD138^+$/$CD38^+$ cells), through Ficoll-Paque (GE Healthcare Life Sciences) density centrifugation. Isolated cells were directly used in cytotoxicity assays or cryopreserved in liquid nitrogen until use. All primary samples were obtained after informed consent and approval by the institutional medical ethical committee.

iNKT cell culture

Isolated iNKT cells were cultured in Yssels'[48] IMDM medium (Gibco) supplemented with 2% human AB serum (Sanquin), 100 IU/ml sodium penicillin, 100 µg/ml streptomycin sulphate and 2 mM glutamine. Additonal cytokines were added: rhIL-7 (10 ng/ml) (Peprotech) and rhIL-15 (10 ng/ml) (Peprotech). iNKT cells were cultured in 48 or 24 well plates and were split 1:2 once a cell density of 2x10^6/ml cells was reached.

Monocyte-derived dendritic cells

Isolated monocytes were cultured in RPMI1640 stimulated with 20ng/ml IL-4 (R&D systems) and 1000U/ml GM-CSF (R&D systems) cultured in RPMI-1640 (Thermo Fisher) + 10% FBS (Invitrogen) + antibiotics (100 IU/ml sodium penicillin, 100 µg/ml streptomycin sulphate) for 5 days, followed by DC maturation with 100 ng/ml a-GalCer and 100 ng/ml LPS (Sigma) for 2 days.

Cell lines

Unmodified or luciferase (Luc-GFP)-transduced human MM cell lines, UM9 and MM1.S (+/-CD1d) were previously described[37,49]. The cell lines cultured in RPMI-1640 (Thermo Fisher) + 10% FBS (Invitrogen) + antibiotics (100 IU/ml sodium penicillin, 100 µg/ml streptomycin sulphate). Phoenix Ampho cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher) + GlutaMAX, 10% FBS (Invitrogen) + antibitotics.

Flow cytometry

Flow cytometry was performed on a LSRFortessa instrument (BD). Viable cells were determined with live/dead cell marker (LIVE/DEAD® Fixable Near-IR; Life Technologies L10119). Transduction efficiency and associated CAR expression was measured with an APC conjugated antibody towards LNGFR (CD271) (Biolegend) for CAR-4-1BBz-LNGFR and APC antibody 4-1BBL (CD137L) (Biolegend) for CAR-28z-4-1BBL. CAR-28z-dsRed were measured in the PE-CF594 channel to detect dsRed. Additional antibodies used for weekly iNKT phenotyping: CD3, CD57 (BD Bioscience), Vα24, Vβ11 (Beckman Coulter), PD-1 and CD39 (Biolegend) and for cytotoxicity assays: CD3, CD19, CD38, CD56 and CD138 (BD Biosciences). Flow cytometry data were analyzed using the FACS Diva 6.1 software.

Flow cytometry-based cytotoxicity assay

Serial dilutions of mock or CAR transduced iNKT cells were incubated with target cells for 16-24 hours. To distinguish them from effector cells, target cells were pre-stained with 0.5 μM Violet tracer (Thermo Fisher). After addition of Flow-Count™ Fluorospheres (Beckman 7547053) cells were harvested and stained for different CD markers (see section flow cytometry) to distinguish different cell subsets. Viable cells were then quantitatively analyzed through Flow-Count-equalized measurements. Percentage cell lysis was calculated as follows and only if the analyzed target cell population contained >500 viable cells in the untreated samples. % lysis cells = (1 − ((# viable target cells in treated wells/# of beads) / (#viable target cells in untreated wells/# of beads))) × 100%.

Bioluminescent Imaging based cytotoxicity assay

Serial dilutions of CAR T cells were incubated with the Luc2-GFP-transduced human malignant cell line UM9. The luciferase signal produced by surviving UM9 cells was determined after 16-24 hours with a GloMax® 96 Microplate Luminometer (Promega) within 15 minutes after the addition of 125 μg/mL beetle luciferin (Promega). % lysis cells = (1 − (BLI signal in treated wells / BLI signal in untreated wells)) × 100%.

Cytokine measurements

To determine the production of IL-2, IL-4, IL-6, IL-10, IL17A, TNF and IFN-γ in the cell free supernatants of CAR T cells stimulated with indicated target cells, we used a Cytokine Bead Array (CBA) Human Th1/Th2/Th17 cytokine kit (BD) according to the manufacturers' protocol.

Statistical analysis

Statistical analyses were performed using Graphpad Prism software version 7.0. For normal distributions, parametric student's t-tests were used. In analyses where multiple groups were compared, either a parametric ANOVA with bonferroni posthoc test or nonparametric

Kruskal-Wallis test was used with subsequent multiple comparison. A p value <0.05 was considered significant.

RESULTS

Isolation and transduction of invariant NKT cells

iNKT cells were isolated from healthy donor PBMCs using magnetic cell sorting. Directly after cell sorting the iNKT purity was determined by flow cytometry, based on the double positive expression of Va24 paired with Vβ11 (Fig. 1A). Following cell sorting, isolated iNKT cells (~50.000 cells) were stimulated and transduced with the affinity-tuned CD38-CAR (CARB1), containing three different costimulatory domains (4-1BB-CD3z, CD28-CD3z +/- 4-1BBL), as schematically depicted in Figure 1B. The low affinity nerve growth factor receptor (LNGFR), dsRed or 4-1BBL, which were co-expressed in the three different CAR constructs were used to determine transduction efficiency, and used as a surrogate for CAR expression, as previously validated[38]. The transgene expression on iNKT cells was determined by flow cytometry using the appropriate markers (Fig. 1C). The mean transduction efficacy of iNKT cells was ~50% (range: 25-75%, n=3). The iNKT purity was determined 4 days post-transduction (supplementary Fig. S1).

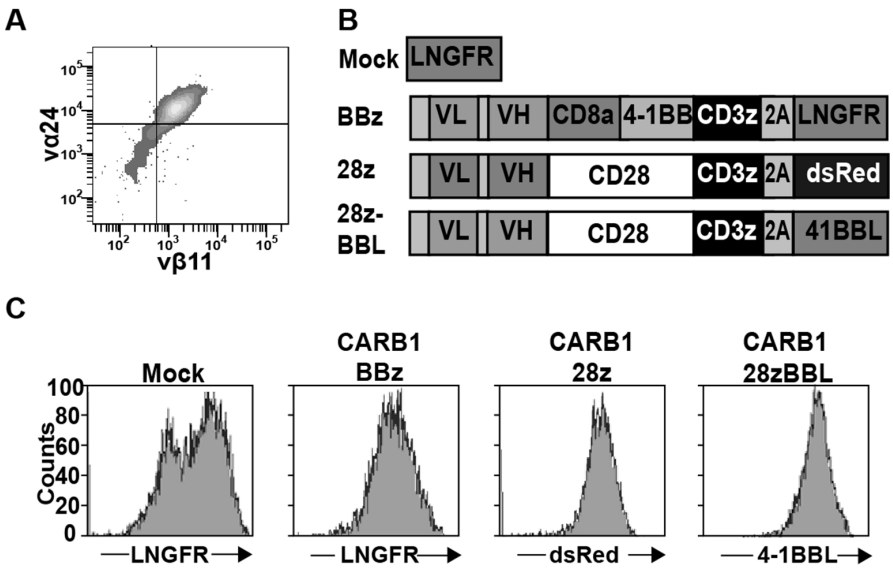

Figure 1. iNKT cell isolation and CAR expression. (A) The magnetic cell sorting with iNKT specific beads resulted in an iNKT (Vα24+/Vβ11+) purity of ~70%. (B) Schematic overview of different constructs used for CAR transduction, transduction efficiency markers LNGFR, dsRed or 4-1BBL are separated by a P2A sequence. (C) Flow cytometry histogram illustrating the marker expression LNGFR and 4-1BBL as detected by APC-conjugated antibodies or by constitutive dsRed expression on the iNKT cells. Data are representative of independent transductions in iNKT cells of three different donors.

CD38-CAR iNKT cells show CD38 and CD1d-dependent cytotoxicity

CD38-CAR transduced iNKT cells were evaluated for their anti-tumor cytotoxicity through their endogenous CD1d-responsive TCR as well as through the CD38-specific CAR, by making use of two CD38$^+$ MM cell lines, UM9 and MM1s (supplementary fig. S2), of which the MM1s cells had also a CD1d transduced variant (Fig. 2A). The CD38$^+$CD1d$^-$ UM9 cells were lysed only in a CD38-dependent manner; there was no NK-cell like lysis or lysis via the endogenous TCR, since the control/mock transduced iNKT cells did not lyse UM9 cells at all. Interestingly, unlike in T cells, where the expression of the CD28 costimulatory signal significantly improves the cytotoxic activity (manuscript submitted), the different types of costimulatory domains did not alter the cytolytic capacity of the CD38-CAR iNKT cells. MM1s cells were also specifically lysed by CD38-iNKT cells, but a much higher background lysis by mock transduced cells was observed, suggesting NK-like lysis activity of iNKT cells to this cell line. Nevertheless, lysis of CD38$^+$CD1d$^+$ MM1s cells by CD38-CAR iNKT cells was still much more effective, close to a 100%, presumably due to the combinatorial effect of CAR-mediated lysis, NK-cell like lysis and lysis via the endogenous TCR (Fig. 2B right panel).

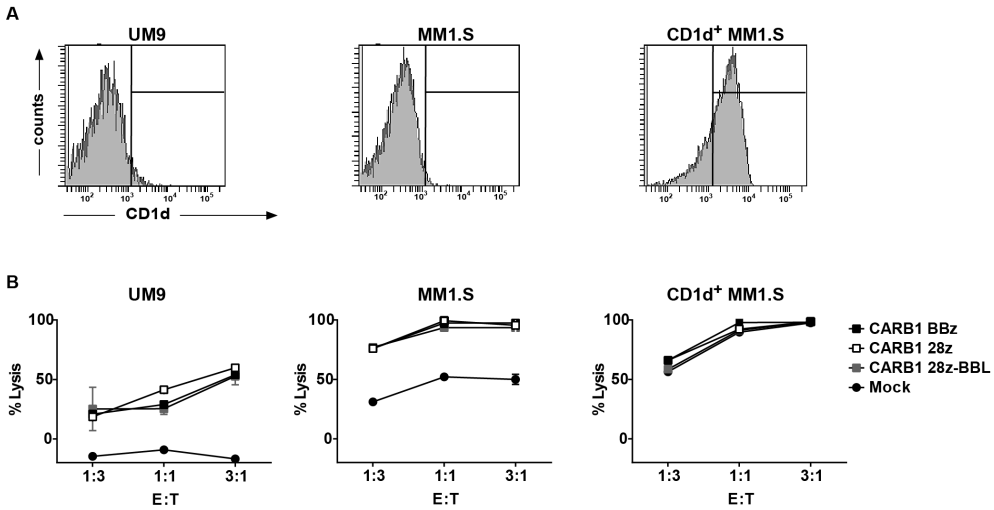

Figure 2. CD38 and CD1d-depedent cytotoxicity towards cell lines. (A) Flow cytometry histograms depicting CD1d expression on CD38$^+$/CD138$^+$ UM9, MM1.S and CD1d-transfected MM1.S cells (supplementary fig. S2). (B) Low affinity CD38-CAR T cells were co-incubated with luciferase-transduced MM cell lines UM9, MM1.S and CD1d-transfected MM1.S for 16 hours. Lysis was calculated relative to untreated wells, measured by luminescence signal on a GloMax Luminometer or flow cytometry based cytotoxicity assay. (●) indicates Mock (□) indicates CARB1-BBz, (■) indicates CARB1-28z, (■) indicates CARB1-28zBBL, n=2 in each condition, mean value +/- SEM.

Maximal on-tumor and minimal off-tumor effects of CD38-CAR iNKT cells

To study the effect of CD38-CAR iNKT cells on primary malignant cells we conducted flow-based cytotoxicity assays on whole bone marrow mononuclear cell (BM-MNC) samples from MM patients. Since BM-MNC samples contain both $CD38^{high}$ MM cells and $CD38^{int}$ healthy cells, this assay system allows us to study the cytotoxic activity of CD38-CAR iNKT cells towards $CD38^{high}$ MM cells in addition to off-tumor effects on $CD38^{int}$ healthy hematopoietic cells[37,38] (Fig. 3A and B). In three samples derived from a patients with $CD1d^-$ MM cells (Fig 3B), the MM cells were not killed by mock transduced cells but effectively lysed by CD38-CAR iNKT cells. Consistent with all our previous studies, the cytotoxic activity of CD38-CAR iNKTs was exclusively directed against $CD38^{high}CD138^+$ MM cells (Fig. 3A and B), while $CD38^{int}CD138^-$ normal hematopoietic cells remained largely intact (Fig. 3A, 3B right panel). This indicated that the optimized CAR affinity was preserved when inserted into iNKT cells. In another sample where MM cells expressed CD1d (Fig. 3C left panel), there was already a significant level of lysis by mock transduced iNKT cells (Fig 3C middle panel), indicating CD1d dependent reactivity, but lysis levels increased substantially when the cells were transduced with CD38-CARs, thus indicating the dual antigen specific targeting of $CD1d^+$ $CD38^+$ MM cells by CD38-CAR-iNKT cells in this sample (Fig. 3C middle panel), while not harming healthy MNCs (Fig. 3C right panel).

The cytokine profiles of CD38-CAR iNKT cells

To further study the other relevant CD38-dependent functions of CD38-CAR iNKT cells, we analyzed their cytokine production profile in response to $CD1d^-$ BM-MNC of $CD1d^-$ MM patients. There were low levels of IL-6 measurable in the supernatant of BM with and without effector cells (Fig. 4, panel IL-6) and very low to undetectable levels of IL-2 (Fig. 2 panel IL-2) and IL-10 (supplementary Fig. S3). Significant levels of the pro-inflammatory Th1-like cytokines IFN-γ and TNF were predominantly produced by CD38-CAR iNKT cells harboring a 4-1BB costimulatory domain (Fig. 4), while the CARs with a CD28 costimulatory domain produced low but slightly more IL-4 and IL-17a as compared to CARs with 4-1BB domain.

CD38-CAR dependent expansion of CD38-CAR iNKT cells

After determining the cytotoxic activity and cytokine profile of CD38-CAR iNKT cells we evaluated their *ex vivo* expandability, since the feasibility of adoptive immunotherapy with this rare T cell subset depends on the generation of large numbers that cannot be isolated from the peripheral blood of patients. To this end, we first evaluated the expansion rates of CD38-CAR iNKT cells in a CD38-dependent manner by stimulating them weekly with the irradiated $CD38^+$ but $CD1d^-$ UM9 cell line (Fig. 5A). Supporting cytokines IL-7 and IL-15 were added to the cultures. As depicted in Figure 5A, the cell cultures were initiated with very low numbers of iNKT cells isolated from buffycoats. Their purity was around 80%

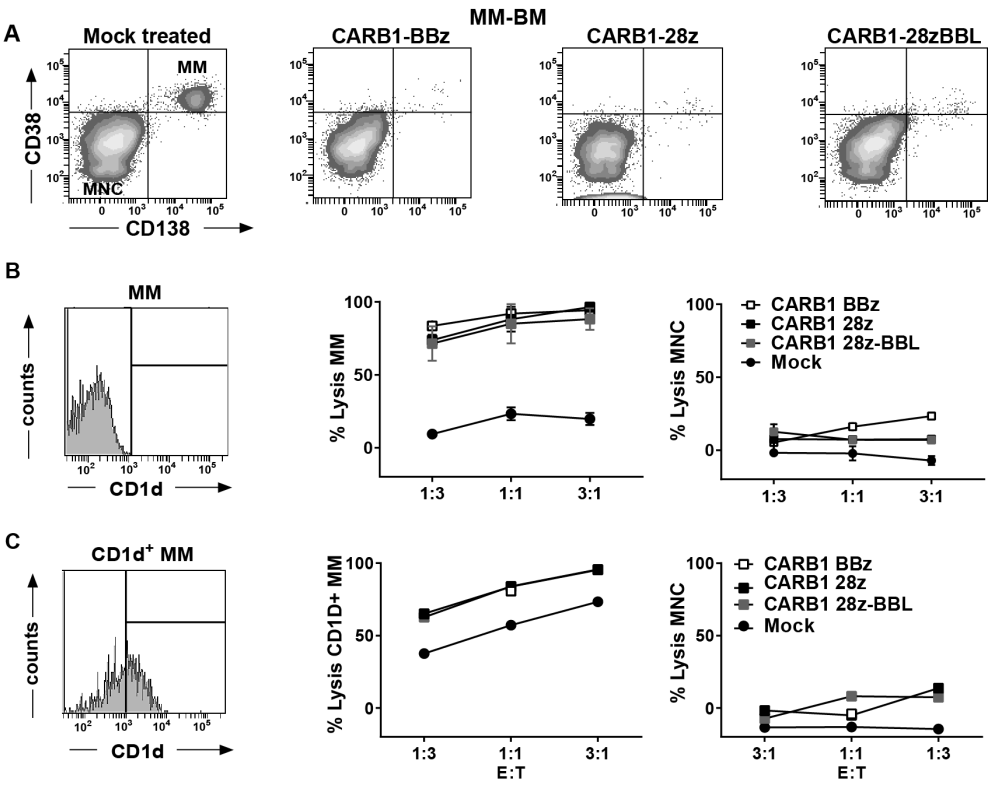

Figure 3. CD38-CAR iNKT cells show on-tumor and minimal off-tumor effects towards MM and healthy hematopoietic cells in whole bone marrow samples. (A) Representative flow cytometry density plots depicting a bone marrow (BM-MNCs) sample of an MM patients with 30% MM cells and after co-incubated with mock or CD38-CARB1 (costimulation BBz, 28z and 28zBBL) iNKT cells for 16 h. (B) Flow cytometry histogram illustrates absence of CD1d expression on $CD138^+/CD38^+$ MM cells in patient's bone marrow and resulting lysis of $CD138^+/CD38^+$ cells (MM) (middle panel) and total MNCs (right panel) n=3 in each condition, mean value +/- SEM. (C) Flow cytometry histogram illustrating expression of CD1d on $CD138^+/CD38^+$ MM cells (left panl) and resulting lysis of MM cells (middle panel) and total MNCs (right panel). n=1, mean value. The flow cytometry assays were conducted with different E:T (E:BM-MNC) ratios as indicated. (●) indicates Mock (□) indicates CARB1-BBz, (■) indicates CARB1-28z, (■) indicates CARB1-28zBBL. The % lysis calculated as described in the methods section.

at day 7 (Fig. 5B). The CD38-CAR iNKT cells vigorously proliferated upon stimulation with UM9, with total CAR^+ iNKT cell yields around 10^9 cells (fold expansion $>10^4$) at the end of the experiment at day 42 (Fig. 5A). Mock-transduced cells proliferated to a much lower extent (10 fold), revealing the significant contribution of CD38-specific stimulation in the exponential growth of CD38-CAR iNKT cells. Interestingly, at the end of cultures (day 42), the % of iNKT cells in cultures transduced with 4-1BBz containing CARs remained exactly the same as at day 7 (± 80%), while the percentage of iNKT cells in other cultures

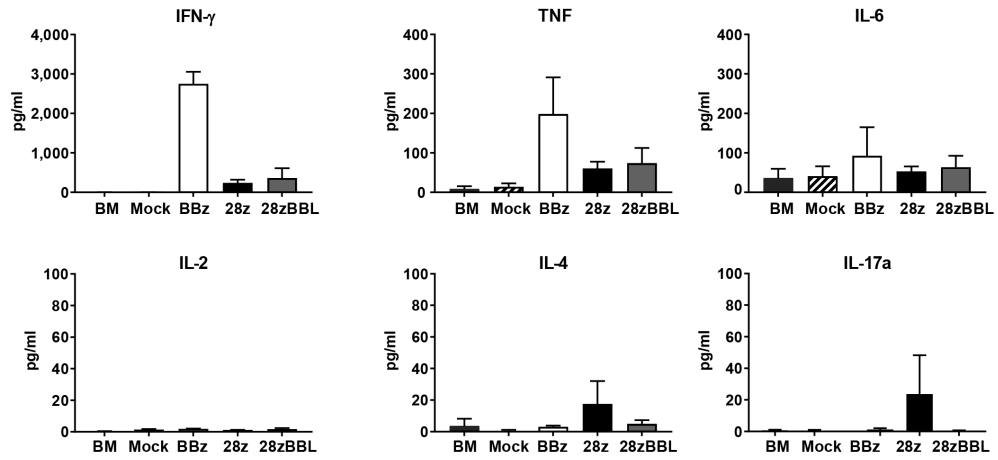

Figure 4. CAR-transduced iNKT cells display a Th1 like cytokine profile. Twenty-four hours after co-incubation with MM-BM samples, cell supernatants were harvested to measure cytokine secretion (E:T ratio 3:1) with a flow cytometry-based assay. Graph shows the secretion of IFN-γ, TNF, IL-6, IL-2, IL-4 and IL-10. (n=3 each condition), n=3, incubated with different patient samples, mean +/- SEM, * indicates p value <0.05, *** <0.001 using parametric ANOVA and subsequent multiple comparison.

diminished significantly, indicating that the 4-1BB provided the most suitable costimulatory signals for CAR-iNKT cells.

Since the cells were cultured continuously for longer than 2 months, we analyzed their phenotype, including their CD4/CD8 ratio and important exhaustion and activation markers (Fig. 5C and D). These analyses revealed that the initial phenotype of all CD38-CAR-iNKT cells was predominantly $CD4^+$, but the 4-1BB costimulatory domain skewed the CAR-iNKT cells towards a double negative phenotype. In contrast, the CD28 costimulatory domain forced them to develop into a more $CD4^+$ phenotype (Fig. 5C). Perhaps more importantly, while CD38-CAR-iNKT cells containing the CD28 costimulatory domain expressed high levels of PD-1 during the whole culture period, iNKT cells containing the 4-1BB costimulatory domain expressed no or low levels of this immune checkpoint molecule. (Fig. 5D, left panel). In addition, expression of CD57, a molecule associated with exhaustion/ senescence[41–44] was virtually absent in all CAR-transduced iNKT cells in contrast to mock transduced cells (Fig. 5D, middle panel). Reversely, CD39 molecule expression, which is thought to regulate activation of NKT cells[45,46], was positive on all CAR transduced, but not on mock transduced iNKT cells (Fig. 5D right panel). These results indicated that long term cultures of specifically CAR iNKT cells driven by 4-1BB costimulation did not induce exhaustion or senescence, which are considered important parameters for the *in vivo* survival and persistence of *ex vivo* cultured T cells.

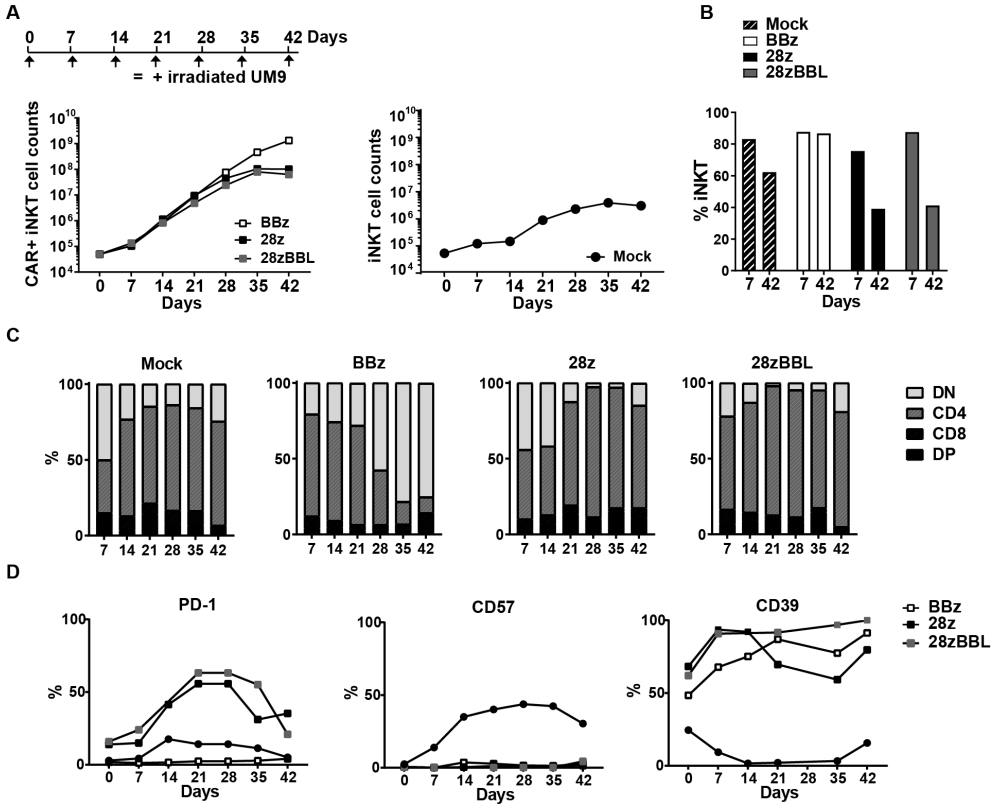

Figure 5. CD38-CAR induced expansion, activation and exhaustion of CD38-CAR-iNKT cells. CD38-CAR iNKT cells with different costimulatory domains (A) and mock transduced cells (B) were co-cultured with irradiated CD38^{+}UM9 cell line at E:T ratio of 3:1, starting one week after transduction and re-stimulated weekly. IL-7 (10 ng/ml) and IL-15 (10 ng/ml) were added to iNKT cell cultures evert 2-3 days. Depicted is the stimulation scheme (insert A) total number of CAR^{+} iNKT cells (A) the total cell counts of mock transduced iNKT cells (B) and the % of iNKT cells in the culture at day 7 and day 42 (C). Upon weekly exposure to CD38^{+} UM9 cells, phenotype of iNKT cells were determined by flow cytometry. Depicted is the % double positive (DP), CD4+, CD8+ or double negative (DN) cells in each culture (D) the % of iNKT cells expressing activation/exhaustion markers PD-1, CD57 and CD39 (E). Similar results were observed in a second independent experiment n=2.

TCR-mediated proliferation of long term expanded CD38-CAR iNKT cells

Finally, we evaluated whether the CD38-CAR iNKT cells could be re-stimulated via monocyte-derived dendritic cells (moDCs) after a long expansion period, because such a strategy could be instrumental to *in vivo* boost the CD38-CAR-iNKT cells after administration into patients. To determine the best timing and stimulation method we stimulated them at different time points with unloaded or a-GalCer loaded DCs, which also express the CD38 molecule. As expected, mock-transduced cells proliferated only upon stimulation with

loaded DCs in an a-GalCer dose-dependent manner (Fig. 6A and B). While CAR-iNKTs containing the CD28 domain did not expand well upon DC stimulation, the CD38-CAR iNKT cells containing the 4-1BB costimulatory domain expanded 3 (at day 14) to 6 fold (at day 21) upon stimulation with antigen unloaded and loaded DCs. Nevertheless, there was a slight advantage of stimulating them with a-GalCer loaded DCs only at day 14, but not at day 35 (Fig. 6A and B). Overall, these results indicated that long term cultured CAR-iNKT cells can be boosted by DCs, but a-GalCer loading did not per se improve their proliferation. Additionally, we compared the phenotype of iNKT cells stimulated either with UM9 cells or with α-GalCer loaded or unloaded DCs at day 14 (Fig 6 C, D). These analyses revealed that CAR negative, mock transduced iNKT cells were skewed towards a CD4 phenotype, expressed higher levels of PD-1, CD39 and decreased their CD57 in an α-GalCer dependent manner. In CAR positive iNKT cells however, the different modes of stimulation did not alter the CAR percentage (supplementary fig. S4) CD4/CD8 phenotype (Fig. 6C), or expression of PD-1, CD57 or CD39 levels on (Fig. 6D) again indicating that a) CAR iNKT cells can be stimulated with no alterations either with $CD38^{+}$ tumor cells or with αGalCer loaded or unloaded DCs and b) the 4-1BB costimulatory domain is the best choice to keep PD-1 expression low in CD38-CAR-iNKT cells.

DISCUSSION

Over the past decade, several studies have convincingly demonstrated the anti-tumor potential of CD1d-restricted iNKT cells[22,32] against different solid and hematological tumors[28,34–36]. It has been shown that iNKT cells not only produce cytokines promoting T-cell activation and NK cell transactivation but also effectively kill CD1d positive tumor cell lines. Although iNKT cells are very low in frequencies in human peripheral blood, the discovery that they can be exponentially expanded *ex vivo* or *in vivo* through stimulation with a-GalCer loaded dendritic cells (DCs), prompted the design of several strategies to utilize iNKT cells in cancer treatment. In MM, however, although CD1d is highly expressed in premalignant and early disease, its expression is reduced and lost eventually in the advanced stages, posing a potential drawback for iNKT-based immunotherapies. In this study, we believe to have successfully tackled this drawback by endowing iNKT cells with CD38-CARs, which can- due to their attenuated affinity- selectively target $CD38^{high}$ MM cells. CD38-CAR transduced iNKT cells effectively redirected the killer functions of iNKT cells toward MM cells, even in the absence of CD1d expression. Moreover, we have observed that CD1d expressing MM cells, were even more effectively killed through CAR- and TCR mediated dual-antigen recognition by iNKT cells. Importantly, CD38-CAR iNKT cells did not mediate any significant toxicity toward normal hematopoietic cells which express intermediate levels of CD38. We thus demonstrate that iNKT cells are excellent carriers for these therapeutically relevant CD38-CARs for the adoptive therapy of MM.

To determine the best CAR costimulatory domain in iNKT cells, it is relevant to compare our study with our recent findings with T cells and with a recent study, in which

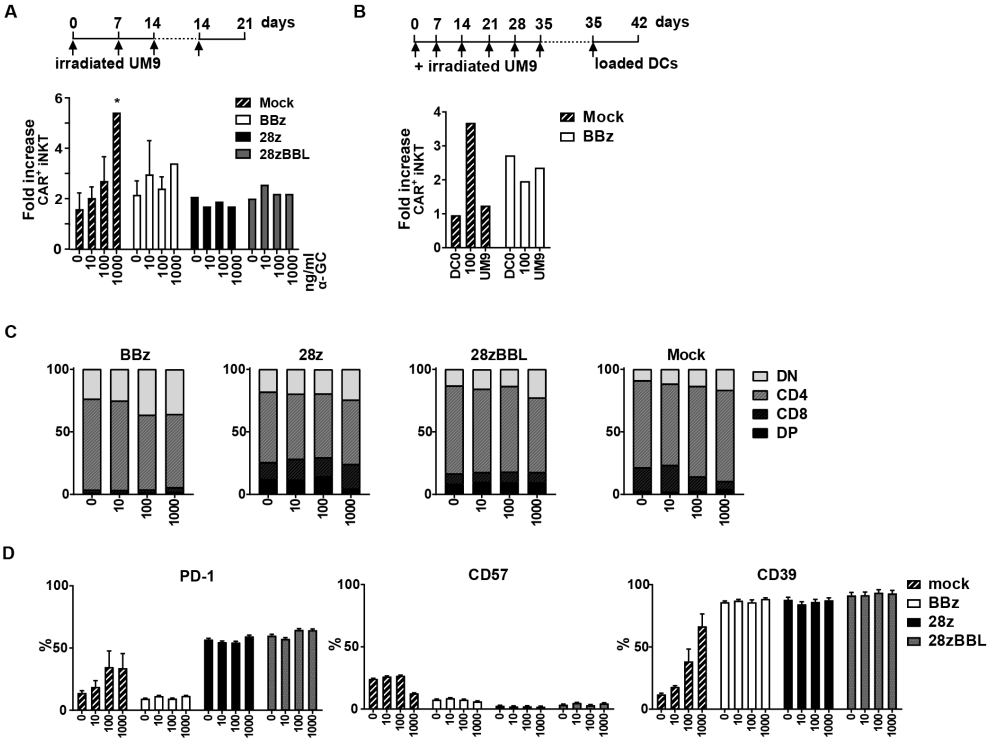

Figure 6. α-GalCer loaded DC induced expansion, activation and exhaustion CD38-CAR-iNKT cells. Depicted is the culture scheme (upper panels) and the proliferation (lower panels) of CD38-CAR iNKTs stimulated with CD38$^+$UM9 cells followed by stimulation with unloaded or aGalCer loaded DCs at 14 days (A) or at 35 days (B) of culture. Number 0, 10, 100 and 1000 indicate the dosage (ng/ml) of α-Galcer. At the indicated times, iNKT cells were co-cultured with DCs (n=2 of separate experiments +/- SEM). Bar graphs depict the fold increase in CAR$^+$ iNKT cell numbers one week after DC culture. One week after DC culturing, phenotypical features of iNKT cells were determined by flow cytometry. (C) depicts the % double positive (DP), CD4+, CD8+ or double negative (DN) (D) depicts the % of iNKT cells expressing activation/exhaustion markers PD-1, CD57 and CD39. Samples depicted n=2 +/- SEM. * indicates p value <0.05 using parametric ANOVA.

CARs with different costimulatory domains were also used to transduce iNKT cells. First of all, in T cells, we and others have recently found that including a CD28 costimulatory domain enhances the cytotoxic activity of CAR T cells compared to 4-1BB signaling. Inducing additional 4-1BB signaling, through the separate expression of a 4-1BBL molecule, in CAR T CD28z cells specifically enhanced the proliferative capacity and T cell persistence[40] (manuscript submitted). However, in iNKT cells we did not observe a beneficial role of expressing the CD28 costimulatory domain, regardless of having an additional separate 4-1BB signaling moiety. In this respect, our results are not entirely in agreement with a recent iNKT CAR study, which suggested that for optimal stimulation of iNKT cells CARs should include both 4-1BB or a CD28 costimulatory domain, although

they demonstrated that the -presumably more relevant- Th1-like polarization was only induced by 4-1BB signaling[19]. In our study, 4-1BB signaling not only induced Th1-like differentiation, but also the best proliferation of iNKT cells. Also cells receiving 4-1BB signals became predominantly double negative, which is consistent with a more cytotoxic phenotype[32,50]. Perhaps more importantly, the iNKT cells receiving 4-1BB signals expressed much lower levels of PD-1 and other exhaustion markers such as CD57[41–44] as compared to the cells receiving CD28 signals, which could readily explain the ability of these cells to continuously expand at least until 44 days upon stimulation with $CD38^+$ tumor cells. Consequently, the cells receiving CD28 signals showed rapid exhaustion and eventually halted proliferation. This disadvantage was not corrected by the separate expression of the 4-1BBL, probably due to a low endogenous expression of 4-1BB on iNKT cells[51] (and data not shown). Although we have not tested a third generation CAR including both 4-1BB as well as CD28 costimulatory domains, we think that 4-1BB signaling provides efficient and sufficient costimulation for optimal expansion and support of the proinflammatory functions of iNKT cells.

In our study we have given specific attention to the *ex vivo* expansion properties of CAR iNKT cells. First of all, we show that CAR transduced iNKT cells can be readily and exponentially expanded via the repeated stimulation of the CAR only, with no need for TCR-mediated stimulation. This CAR specific stimulation does not need to be given by a superior antigen presenting cell; it can be readily provided by tumor cells without induction of dysfunction, exhaustion or anergy. We also show that CAR specific stimulations, which were given by tumor cells, can also be given by DCs during the long term *ex vivo* expansions. As shown in previous clinical trials, the stimulation of iNKT cells via cellular DC vaccines may boost their numbers and activity *in vivo*. Nonetheless, rather surprisingly, we did not find a significant positive effect of loading a-GalCer on DCs to stimulate CD38-CAR-iNKT cells, while this was necessary to expand mock transduced iNKT cells. Although a-GalCer loading of DCs also seemed somehow beneficial for CD38-CAR transduced iNKT cells in the beginning of the cultures, it was not beneficial at the later stages of the iNKT expansion suggesting that the high levels of CD38 expressed by mature DCs was apparently sufficient to provide the most optimal signals for induction of proliferation of CD38-CAR iNKT cells. Extra stimulation via the native TCR may have caused overstimulation, followed by activation induced cell death, a phenomenon demonstrated in CAR transduced alloreactive T cells[12]. In conclusion, our study demonstrates not only the feasibility but also the most optimal conditions for the generation of CD38-CAR-transduced iNKT cells to maximally exploit the therapeutic properties of CD38-CARs and iNKT cells in the battle against MM. Since iNKT cells can be safely used across HLA-barriers and can be exponentially expanded *ex vivo* without losing their functional properties, this strategy holds great potential to apply to a broad patient population, hereby tackling an important drawback of CAR based adoptive immunotherapy strategies.

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DISCLOSURES

There is no relevant potential conflict of interest to disclose.

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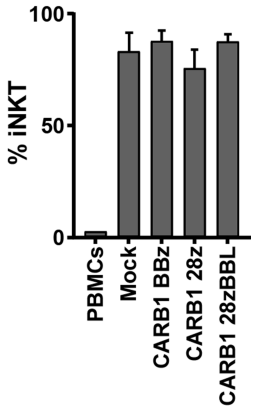
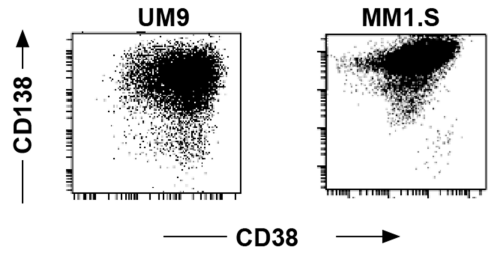
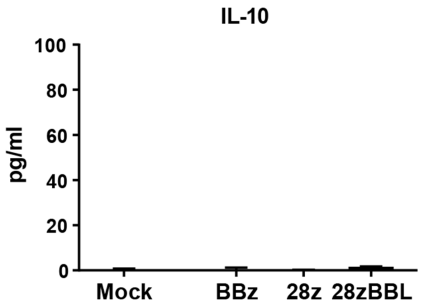
SUPPLEMENTARY MATERIAL



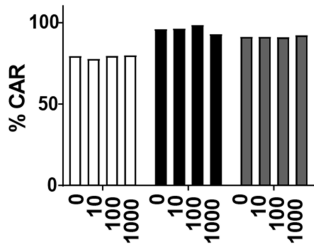
Supplemental Figure 1. Vα24+/Vβ11+ expression on Mock and CAR transduced iNKT cells. After magnetic cell sorting with iNKT-specific beads resulted in a iNKT (Vα24+/Vβ11purity of ~70%, after which cells are transduced with Mock, CARB1-BBz, CARB1-28z or CARB1-28zBBL constructs. Figure depicted n=3 +/- SEM.



Supplemental Figure 2. $CD38^{+}/CD138^{+}$ MM cell lines UM9 and MM1.S cells. Flow cytometry dot plots depicting the expression of both CD38 and CD138 on MM cell lines.



Supplemental Figure 3. Undetectable IL-10 production by Mock and CD38-CAR iNKT cells. 24 hours after co-incubation with MM-BM samples, cell supernatants were harvested to measure cytokine secretion (E:T ratio 3:1) with a flow cytometry-based assay. Graph shows the secretion of IL-10. (n=3 each condition), Data represents n=3, incubated with different patient samples, mean +/- SEM.



Supplemental Figure 4. One week of DC boost did not alter % CAR. Percentage of CAR determined by flow cytometry using monoclonal antibodies staining LNGFR (BBz), dsRed (28z) or 4-1BBL (28zBBL) on CD38-CAR iNKT cells. CAR percentages were determined after DC culture with either 0, 10, 100 or 1000 ng/ml aGalCer.

CHAPTER 7

GENERAL DISCUSSION

Our immune system can effectively clear tumor cells, however the own power of the immune system is not always sufficient to control the tumor growth. Immunotherapies utilize and adjust certain features of the immune system to effectively and specifically attack cancer cells. Currently the most appealing immunotherapy approaches include antibodies, immune modulatory agents or cellular approaches, such as T cell receptor (TCR) or chimeric antigen receptor (CAR) engineered T cells.

Due to the HLA unrestricted fashion of antigen recognition, the built-in costimulatory domains and potent antigen recognition by their single chain variable fragments, CAR T cell therapies are currently considered one of the most powerful approaches in tumor immunotherapy. The production of CAR T cells is also not very complicated. Indeed, over the past years, the number of CAR clinical trials has expanded from a handful to more than one-hundred, and the success of CD19-CAR therapies led to the approval of CD19-CAR T cells as therapy for relapsed B-ALL patients by the FDA in the United States of America[1].

All studies described in this thesis were designed and executed to optimally exploit CAR T cells for the therapy of Multiple Myeloma (MM) and by targeting a tumor-associated but not entirely tumor-specific antigen, the CD38 molecule.

CD38-CAR T CELLS FOR MM

In this thesis, we explored the options of CD38-targeted CAR T cell therapy for MM. The CD38 protein is highly and uniformly expressed on malignant plasma cells, with an approximate ten times higher expression levels as compared to healthy cells[2–4]. The utility of CD38 as an adequate MM-target has been demonstrated by clinical responses seen in patients treated with the CD38-specific human monoclonal antibody daratumumab[5]. Nonetheless, when we started our studies, little was known about the feasibility, efficacy and potential toxicity of targeting CD38 with CAR T cells. Therefore, in **chapter 2** of this thesis, we started with an evaluation on the feasibility and efficacy of targeting CD38 with CAR T cells. To address this, we used the variable heavy and light chain sequences of three different high affinity human CD38 antibodies and generated second generation CAR constructs containing the 4-1BB costimulatory domain. T cells from healthy individuals and MM patients, transduced with these constructs, properly expressed the CD38-CAR on the cell surface and showed CD38-specific proliferation and Th1-like cytokine production, illustrating the feasibility of equipping T cells with CD38-CARs. The CD38-CAR T cells effectively lysed CD38 positive MM cell lines and MM cells in primary patient samples *in vitro*, and demonstrated objective anti-tumor effects in a xenograft model, illustrating the potential anti-tumor efficacy of this approach. However, in this study, we have also noted potential undesired effects due to on-target/off-tumor lysis. First of all, and to our surprise, the CAR T cells themselves were devoid of CD38, which should actually be expressed on all activated T cells. This suggested that they lost the CD38 due to 'self-killing' or 'fratricide' of activated $CD38^+$ expressing fractions, which was also observed in one other CD38-CAR study[6]. Fortunately, however, even deprived of CD38, CD38-CAR

T cells, but also control CD38$^-$ CD19-CAR T cells, appeared to be fully functional, similar to T cells *with* CD38 expression. More importantly however, we observed that CD38-CAR T cells were also cytotoxic towards every type of normal CD38$^+$ cells in the peripheral blood and patient's bone marrow, including NK cells, monocytes, CD38$^+$ fractions of T cells and CD38$^+$CD34$^+$ progenitor cells. Therefore, we focused on defining the potential drawbacks of this approach. Although CD38-CAR T cells eliminated CD38$^+$ fraction of CD34$^+$ hematopoietic progenitor cells, they did not inhibit the forming of granulocytic and myeloid colonies in a colony forming assay, suggesting that normal hematopoietic development would be possible even in the presence of CD38-CAR T cells. This was extended into an *in vivo* model in chapter 3 and 4. Since T cells also appeared to grow normally and function properly in the absence of CD38, we carefully concluded that CD38-CAR T therapy, if fine-tuned properly, could be considered as a clinical treatment.

7 REDUCING OFF-TUMOR EFFECTS OF CD38-CARS

In an ideal therapeutic setting, one would like to maximally exploit the differential target antigen expression levels between tumor cells and normal cells. In fact, CD38 with its extremely high and homogenous expression on MM cells is perhaps the most beautiful example of such a differentially expressed antigen. Its significant higher expression on MM cells compared to healthy cells suggests the possibility to selectively target high expressing cells, if the optimal conditions can be generated. Indeed, other studies demonstrated that healthy cells with lower densities of a tumor-associated antigen (TAA) can be spared by CAR T cells with a lower affinity[7–12], which could avert on-target off-tumor effects.

The feasibility to reduce off-tumor cytotoxicity by affinity-tuned CARs was described in one study by comparing two distinct antibodies, recognizing different epitopes on the same target (e.g. EGFR-antibodies, cetuximab and nimotuzumab)[9]. In other studies, the affinity of existing antibodies were decreased, either by the introduction of mutations or the replacement of human residues in the scFv domain with murine sequences[8,12,13]. However, these studies did not describe a rational approach for generation of candidate scFvs with an optimal target affinity.

Therefore, in **chapter 3** we developed a new technique to generate a large panel of antibodies with different affinities. As previously described[14–18], the exchange of the variable light chain can have a tremendous effect on the affinity of an antibody. Thus, we generated a large panel of CD38 antibodies by variable light chain exchange. This allowed the generation of a large pool of potential antibody candidates, each with different stability and affinity. After extensive analyses, we could subdivide these antibodies into different affinity classes, which led to a selection of 24 candidate scFvs. Functional analysis with CAR T cells bearing the scFvs derived from this selected panel, even with more than a 1000-fold lower affinity, were still functional and tumor selective killers of CD38^{++} MM cells. Simultaneously, we found little or no lysis of CD38$^+$ normal human hematopoietic cells in whole bone marrow cytotoxicity assays. Interestingly, the lower affinity CD38-CAR

T cells displayed less or even no fratricide, suggesting that the long-term exposure to CD38-CAR T cells has no effect on healthy cells, such as the CAR T cells themselves.

In **chapter 3** we also analyzed the effect of CD38-CAR T cells on hematopoietic progenitor cells more thoroughly and for a longer time span in the *in vivo* xenograft scaffold model in which $CD34^+$ progenitor cells with a fluorescent cell label were injected in the bone marrow-like scaffolds. The development of such *in vivo* model is more informative, as other studies merely analyzed the potential toxicity of attenuated CARs only *in vitro* using cell lines[8–10,12] with different target expression levels.

In our *in vivo* model, CAR T cell therapy, even with high affinity CD38-CAR T cells, did not affect $CD34^+$ cells viability. The post-mortem analysis of the scaffolds, however, indicated significant lower numbers of the $CD38^+$ fraction in mice treated with high affinity CD38-CAR T cells. The mock and lower affinity CD38-CAR treated mice displayed similar numbers of CD38-positive cells. This suggested that the treatment with low affinity CD38-CAR T cells would not harm hematopoietic progenitor cells and their outgrowth, while high affinity CD38-CARs could have unforeseen consequences for the hematopoietic development due to $CD34^+/CD38^+$ depletion.

EFFECT OF CD38-CAR T CELLS ON OTHER HEALTHY TISSUE

The results in the above described *in vivo* model suggests the safety of the generated low affinity CD38-CARs. However, the effect on other healthy tissues such as the prostate[19], purkinje cells[20], and lung smooth muscle cells[21], which may express CD38, is difficult to predict as there are no appropriate models to test their susceptibility. Therefore we performed immunohistochemistry studies (data not shown) and found CD38 expression only on (infiltrated) hematopoietic cells in the immunohistochemistry slides. In the light of these data it seems likely that the expression of CD38, on other tissues is much lower than in hematopoietic cells and therefore, these tissues are unlikely to be targets of therapy with attenuated CD38-CAR T cells.

THE IMPORTANCE OF COSTIMULATORY DOMAINS OF CARS

Extensive efforts were made to optimize CAR affinity or adjustment of its extracellular components. However it is also essential to fine-tune the extracellular recognition with intracellular cell signaling as they affect the performance of CAR T cells together. This is evident by the fact that the first generation of CARs, which solely expressed the CD3ζ-chain were not effective. The addition of costimulatory domains such as 4-1BB or CD28 in "second generation" CARs resulted in more viable and persisting CAR T cells.

The effect of costimulatory domains on the clinical efficacy of CAR T cells has been studied extensively. Even though clinical trials with CD28 or 4-1BB-based CD19-CAR T cells have shown comparable responses in patients[22–24], 4-1BBζ-CAR T cells demonstrated an increased longevity, with detectable CAR T cells in the circulation, sometimes even years after infusion[24]. This was in contrast to CD28ζ CAR T cells, with CAR detection for

only several weeks[23]. The better persistence 4-1BBζ CAR T cells could be explained by their better memory phenotype, reduced exhaustion and better metabolism than CD28ζ CAR T cells[25,26]. On the other hand, CD28 costimulation induces a more stringent cytotoxic capacity and more IL-2 secretion[27,28].

Ideally, one would want to combine the positive characteristics of both 4-1BB and CD28 in once CAR. Indeed, such "third generation CARs", with both CD28 and 4-1BB in one fusion protein outperformed second generation CARs in cytotoxicity[29–32], but these constructs were not superior in a clinical setting[33].

Another approach, described in a recent study[28], analyzed many different strategies to provide the optimal costimulatory signals. CAR T cells were transduced with either CD28 and/or 4-1BB or by co-expression of their ligands (CD80 or 4-1BBL). Compared to all other costimulation combinations, T cells transduced with a CD28ζ-CAR, while separately co-expressing a 4-1BBL showed the highest anti-tumor efficacy and accumulation *in vivo* and lower expression of exhaustion markers. This suggested that the CD28ζ + 4-1BBL design provided the optimal combination of costimulatory signaling.

However, we hypothesized that the type of costimulation could affect the cytotoxicity, expansion and persistence of affinity-tuned CD38-CAR T cells in **chapter 4**. In an extensive study, using CD38-CARs with 5 different affinities (K_D range 1.8 - >1915 nM), in combination with 3 different described costimulatory designs (CD28ζ, 4-1BBζ and CD28ζ + 4-1BBL), we confirmed that lowering the affinity of a CAR gradually diminished the cytolytic activity of CAR T cells in a 4-1BB costimulatory design. Interestingly, the cytotoxic activities of CARs did not diminish when the same CARs contained a CD28 costimulatory domain, alone or expressed in combination with 4-1BBL. Importantly, the CD28ζ + 4-1BBL design potentiated even CARs bearing scFvs with immeasurable affinity (>1915nM) and brought them to the functional level of 4-1BB ζ-CARs of higher affinities. The combination of CD28ζ and 4-1BBL provided lower affinity CD38-CAR T cells with a significantly better discriminative capacity *in vitro* and *in vivo*. Furthermore, these cells maintained a central memory phenotype, showed better *in vitro* expansion and improved *in vivo* persistence compared to the other two configurations. Therefore we concluded, based on all data described in **chapter 2, 3 and 4**, that the CD28 signal integrated in the CAR construct combined with a separate expression of 4-1BBL also provided the most optimal activation signals to affinity-tuned CD38-CAR T cells.

SUICIDE GENES AND INDUCIBLE CAR DESIGNS

Increasing the selectivity of the CAR via affinity optimization is an elegant way to improve CAR T cell safety. Though, it may also be possible to achieve this goal by rapid abrogation of the CAR T cell activity in the case of undesired effects. To this end, the most frequently suggested strategies are the use of suicide genes or inducible CAR designs.

Among other strategies (HSV-TK, CD20/EGFR antibody targeting), the use of the apoptosis machinery is a clean suicide method to eliminate cells. During normal

apoptosis, the caspase 9 is dimerized and induces cell death. With an adjusted caspase 9 structure, dimerization can be achieved by the addition of a small molecule (dimerizer), as a safe drug[34–36]. Co-transduction into CAR T cells with such an iCasp9 allows for a selective elimination of CAR+ cells, which can circumvent potential toxicity. Such a suicide gene was tested in the CD38-CAR T cells in **chapter 2** and showed selective killing of iCasp9/CAR transduced T cells, while surrounding cells were left unharmed.

Nonetheless, since a suicide system completely eradicates the therapeutic T cells the application and improvement and correct tuning of on/off switches, can have a great potential to become an effective addition to safe CAR therapy. Several studies propose different innovative adaptations and concepts to generate inducible CAR designs. For instance a synthetic-notch inducible CAR system in which one antigen-recognition domain consists of a synthetic molecule with a Notch core as an artificial transcription factor, which gets cleaved off and transcribes the second tumor-targeting CAR[37,38].

While these and other innovative approaches are in full development, in **chapter 5** we tested the possibility for a transcriptional control of a CD38-CAR using a well-known Tet-On system. In this system the rtTA protein can, upon doxycycline administration, bind to the inducible promoter to control CAR transcription. This method was previously shown to be effective for CD19-CARs[39].

In our study it was demonstrated that we could induce a CAR-mediated T cell cytotoxicity in a dose dependent manner. Especially with a lower dose of doxycycline its removal resulted in a relatively rapid decay of CAR- related off-tumor effects. Or could provide a second level of safety to T cells with an affinity-optimized CD38-CAR, which already displayed a low toxicity profile. We conclude here that an inducible system, like the Tet-on system, may be more advantageous above suicide-genes to control the potential toxicities of CAR T cells without the need to destroy them permanently.

DUAL CAR T CELLS

In this thesis, we have described several strategies towards the improvement of safety of single CD38-CARs, but another attractive approach for increasing the specificity of CARS is dual/split CAR strategy, where CAR T cell activation can only happen when both antigen A and B are recognized by two different CARs. In this concept, tumor specificity is achieved by separating the T cell activation signal from costimulation signal[40–42]. Requirement is that the two selected antigens -although they are not truly tumor specific- are as a combination specifically expressed on tumor cells.

Regarding the targeting of MM, several target cells, such as CD138 or CS-1 could be used in combination with CD38, These combinations are fairly specific for MM cells. Thus it is certainly warranted to test this concept in future studies. Split dual targeting with CD38-CARs may also improve the existing, but less efficient CAR therapies. For example, although BCMA-CAR T cells have proven to be effective, there is a wide variety in the BCMA expression among patients[43,44] and heterogeneity in responses and potential

tumor escape[45]. Potentially, high affinity CD38-CARs, if not used as second generation CARs, could be used as a costimulator to provide a better recognition and a stronger boost for BCMA-CAR T cells to improve the clinical outcome.

TOWARDS UNIVERSAL CAR THERAPIES

Most of the current CAR studies are performed with autologous or HLA-matched allogeneic (donor) T cells. T cells are effective immune cells, with lytic capacity, but are also potentially allo-reactive when used across HLA-barriers. Furthermore, T cells can form an immune memory pool, which is an advantage if the memory response is tumor specific, but can be detrimental if the response is autoreactive. Therefore other immune cells are being considered as alternative carriers for CARs. Recent studies tested NK cells[46], or specific NK cell lines (NK92)[47–49]. Advantages of using NK cell-like immune cells, are their innate properties, the relative short life-span and no/little memory development. In case of an off-tumor cytotoxicity, a short stringent response and thereafter a natural reduction of the engineered cells is potentially safer. But, more importantly, NK cells do not induce graft-versus-host disease (GvHD)[50,51], therefore they may also be potential candidates for a more universal and broader application.

In **chapter 6** we describe a different specific subset of innate-like immune cells, invariant natural killer T (iNKT) cells as carriers of CD38-CARs. This is a specific subset of T cells with NK cell like properties. iNKT cells produce not only have rapid cytolytic function but also harbor an invariant T cell receptor[52], which is not associated with GvHD and even associated with graft-versus-tumor[53] and can be readily triggered by α-GalCer loaded on CD1d molecules[54]. In MM and other tumors, iNKT cells have already been shown to possess therapeutic properties[52,55,56]. Thus by endowing them with an additional CD38-CAR, we have demonstrated that the therapeutic effects of iNKT cells can be maximally exploited and this may be, in the future even possible in an HLA-independent manner. Thus together with all other chapters, this final chapter of this thesis suggests that universal application of CD38-based CAR therapies is not an utopia, but can be more close to the realization with well-designed and systematically executed innovative strategies in the near future

CONCLUSION

Immunotherapy is on its way to become one of the more powerful and specific types of therapy for cancer patients. The introduction of CAR T cells in the field of cellular immunotherapy has proven to be an enormous success and this field is expanding rapidly. To make this type of therapy accessible to more patients, researchers worldwide strive to improve CAR T cell therapy and prevent potential toxicities. These important aspects of CAR T cell development were studied and presented in this thesis: towards a safe and applicable CAR T cell treatment for multiple myeloma.

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CHAPTER 8

ENGLISH SUMMARY &
NEDERLANDSE SAMENVATTING

ENGLISH SUMMARY

Introduction

Our immune system can protect us fantastically well from diseases (viruses, fungi and bacteria), as well as from the development of tumors. But in some cases the immune system fails and tumors can develop. Standard treatments for many forms of cancer are: surgical removal of tumors, radiation and/or chemotherapy. These treatments can be successful, but can also cause damage to the whole body with moderate to severe side-effects.

In the past decades, much attention has been paid towards the improvement of cancer therapy, in order to specifically target tumor cells. To this end, the specificity of the human immune system is used and is called 'immune therapy'. The recent progresses in the development of immunotherapy resulted in: 'Immunotherapy for the treatment of cancer' as break-through of the year published by the scientific journal *Science* in 2013[1]. This was partly due to the great successes with T cells carrying a specific receptor: the Chimeric Antigen Receptor (CAR)[2–4].

Normal T cells have a key role in the defense against tumors, but in order to target T cells against a tumor, they must first be able to recognize a specific tumor protein on the cell[5]. They normally do this via their T cell receptor (TCR) that is present on the T cell surface. However, if recognition of a tumor protein cannot take place by a TCR, a CAR can be preferable. With CAR T cells, recognition takes place via a cleverly synthesized receptor made in the laboratory on the basis of an antibody, which has been made part of the T cell receptor via genetic manipulation[6,7]. This receptor is then called a **c**himeric (consisting of 2 parts: antibody and TCR) and recognizes a surface molecule (also called **a**ntigen) by its **r**eceptor (together CAR) (Figure 1A and C). A CAR gene can be placed on the T cells of a donor or patient using a special technique (transduction), using viruses[8]. The artificial receptor is then 'expressed' on T cells. (Figure 1B). This creates a strong combination on the CAR T cell: one part of the receptor for recognition by the antibody part, another part for signaling to kill the target (the tumor cell) (Figure 1C).

The subject of my thesis is to develop a safe and effective CAR T cell therapy for multiple myeloma.

Multiple myeloma and CD38

Multiple myeloma (MM) is a form of bone marrow cancer caused by malignant white blood cells (plasma cells). This type of cancer covers 10% of all blood and bone marrow cancers in adults worldwide. Patients have symptoms such as bone pains and fractures, anemia, kidney failure and reduced immunity. In the past 30 years, the treatment of MM has greatly improved, with enhanced survival rates[9]. Recent studies, partly carried out by our group, have shown that the daratumumab antibody works very efficient in MM patients[10–12]. Daratumumab is directed towards CD38, a molecule that is present in large numbers (high expression) on MM cells. In this thesis we study the possibility to target CARs against CD38.

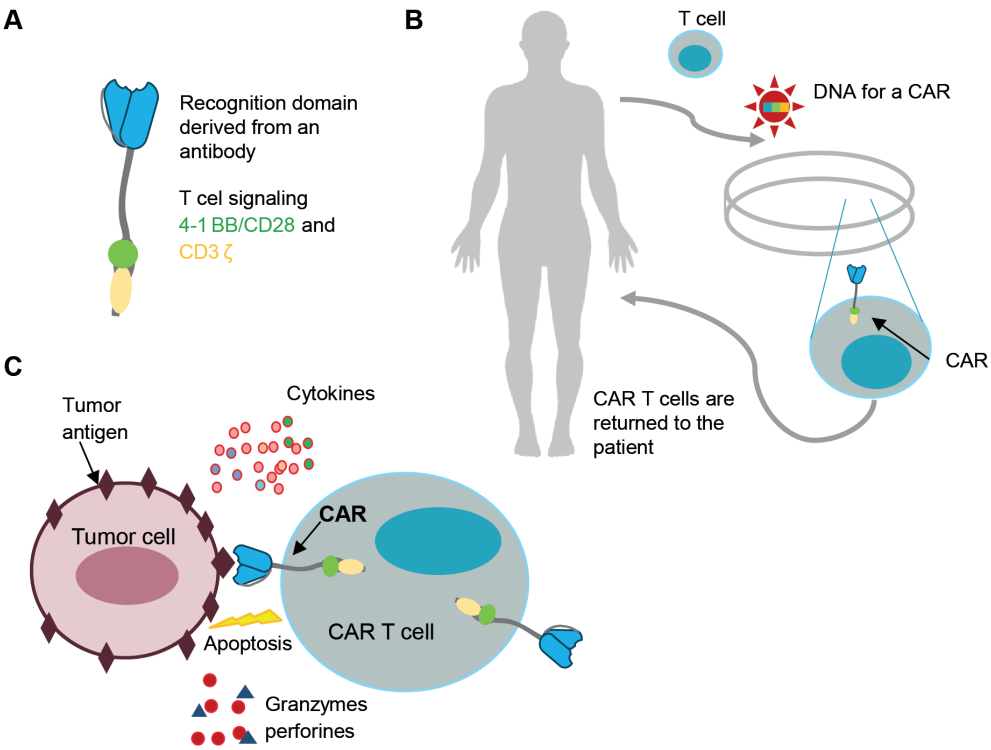


Figure 1. (A) Schematic overview of a chimeric antigen receptor structure with recognition domain derived from an antibody and one –or more- signaling domains (4-1BB of CD28 and CD3ζ). (B) Application of CAR T cell therapy. T cells are isolated from patients' peripheral blood, generated into CAR T cells in the laboratory. CAR T cells are returned to the patient via infusion. (C) The CAR T cell can kill the tumor cell via induction of apoptosis and secreting cytokines and granzymes/perforines.

Chapter 1 of this thesis provides a general overview of CAR design, ways to improve and the current CARs tested for MM.

CD38-CAR T cells

Because CD38 is highly expressed on MM cells, we have used the recognition parts of CD38 antibodies and generated different CD38-CARs, described in chapter 2. Healthy T cells from donors were used to produce CD38-CAR T cells. We tested these CD38-CAR T cells on bone marrow samples from patients and they proved to be extremely effective in eliminating MM cells. This was extended in a MM mouse model, which was developed by our group[13]. Indeed, the CD38-CAR T cell-treated mice showed reduced tumor growth compared to the control group.

However, what we also saw was that CD38, although to a much lesser extent, is also expressed on healthy cells, mainly on white blood cells. Although these CD38-CAR T cells appeared to be very effective against MM cells with high CD38 expression, they also

showed a response against healthy cells with a lower CD38 expression. This is obviously not favorable, since such CD38-CAR T cells would also eliminate healthy cells of patients.

More specificity with a fine-tuned affinity

Although the healthy white blood cells also express CD38, the amount is much lower than on MM cells[11]. That is why we wanted to make a CD38-CAR that could distinguish between CD38-high versus CD38-low. To this end, the CD38-CAR binding with CD38 has to be weakened, so that binding only occurs when many CD38 molecules are present. This "strength of binding" is also called affinity

The first CD38-CARs from **chapter 2** were selected for their strong affinity towards CD38. In **chapter 3** we therefore look for a suitable CD38-CAR with a lower affinity for CD38. As discussed in the introduction, a CAR consists of an antibody recognition domain. To this end, we tested a whole panel of new CD38 antibodies for their affinity to CD38. We found many antibodies which recognized CD38, but with a 10 to more than 1000 times lower affinity than the original strongest antibody. From the selected antibodies we generated new low affinity CD38-CAR T cells. The affinity of these new CD38-CAR T cells appeared, as expected, to distinguish between CD38-high on MM cells and CD38-low on healthy cells. Also in the MM mouse model, there was no difference between high and low affinity CD38-CAR T cells in the inhibition of tumor growth. But, very important, also in the mouse model, the low affinity CD38-CAR T cells did not show a negative effect on healthy white blood cells, while the high affinity CAR T cells did. We were therefore able to increase their ability to distinguish by lowering the affinity of the CD38-CAR.

Effect of costimulation on low affinity CD38-CAR T cells

The low affinity CAR T cells compared in **chapter 3** all had the same T cell signaling domains to activate the cell. However, these signaling domains can have a great influence on the T cells and provide different characteristics. CARs are composed of an antibody and a part of the T cell receptor (TCR), CD3ζ domain (Figure 1A). A lot of research has focused on the addition of costimulation to CARs. There are many flavors of this costimulation, the most studied costimulations are: 4-1BB and CD28. Roughly 4-1BB gives the signal to let T cells divide (proliferate), while CD28 is much more deadly for the tumor cell[14,15]. In chapter 2 and 3 all CARs were generated with a 4-1BB signal. In **chapter 4** we were interested in the replacement of the 4-1BB for a CD28 part and also the effect of both signals, CD28 + 4-1BB. It was immediately clear that the same low affinity CD38-CARs with CD28 instead of 4-1BB indeed eliminated relatively more tumor cells. The low affinity CARs that we had chosen in chapter 3 were suddenly much stronger when costimulation was changed. To protect healthy cells, the optimal CAR needed an even lower affinity.

Although CD28 provided stronger CARs, the addition of 4-1BB signaling also positively influenced the CARs because they were able to proliferate and were less exhausted. Also in the mice, the CAR T cells with both costimulations (CD28 + 4-1BB) also persisted better

in mice[14]. The remaining T cells were still able to suppress tumor growth. The low affinity of the CD38-CAR T cells still protected the healthy cells in all cases.

Thus, the type of costimulation had a strong influence on the performance of low affinity CARs.

Controlling a CAR

In addition to reducing the affinity from chapter 3 and 4, we can also influence the effect of CAR T cells by switching the CAR expression on and off.

In **chapter 5** we describe the possibility of regulating gene expression, the transcription of the DNA code for a CAR, with an antibiotic. In clinical practice, this means that the CAR is only expressed by the T cells when the patient is treated with a medicine (antibiotic). By stopping administration, the expression is reduced again and the possible 'danger' of the CAR will disappear. In our experiments we indeed saw that within a few hours the expression of the CAR could be measured and the expression level was also lower at a lower dose of the antibiotic. By removing the antibiotic, by washing the cells, expression slowly decreased. We also observed that the cells with low expression had a preference for the MM cells and not the healthy cells. This suggests that both the lower affinity, but also the regulated expression levels, propose an opportunity to reduce any adverse effects of CAR T cells.

A universal CAR

The current practice of CAR T cell therapy is shown in figure 1B, blood cells are taken from the patient and returned after the CAR has been implemented. This is generally a labor-intensive, expensive, but also safe way of T cell administration. To apply CAR therapy more broadly, other cells may offer a solution. T cells have the good characteristic of recognizing small cell differences, but also increase the chance of graft-versus-host disease, which means that T cells from a donor can attack a patient. One way of preventing this is to use other cells, for example (Natural Killer) NK cells.

In **chapter 6** we looked at a separate small subset within the white blood cells: the invariant NKT cells (iNKT cells). This group of cells share properties of both NK cells and T cells[16]. They have a rapid killer function, but also a T cell receptor, like T cells. In the 'invariant' NKT cells, all T cell receptors are identical and all recognize small structures (glycol-lipids) presented in the molecule CD1d[17]. Tumor cells such as MM can present a lipid in CD1d that would activate T cells[18]. When we introduce a CD38-CAR in the iNKT cells, these cells can recognize two molecules: lipids in CD1d and CD38. This allows us to use both receptors to grow iNKT cells and kill tumor cells. Importantly, iNKT cells, due to their CD1d-restriction, do not induce and alloreactive response can even protect from graft-versus-host disease[19]. With this strategy a broader patient population can be treated with the same cell product.

Conclusion

Immunotherapy is a promising field within cancer therapy. Clinical studies with CAR T cells have shown how strong this anti-tumor weapon is. In order to make CAR T cell therapy accessible to more patients, we focus on improvement and the reduction of toxicity. These important aspects of the development of CAR T cells have been studied and presented in this thesis: towards a safe and applicable CAR T cell treatment for MM. In **chapter 7** the results of this thesis are summarized and discussed, furthermore suggestions are made to further research.

NEDERLANDSE SAMENVATTING

Inleiding

Ons immuunsysteem kan ons fantastisch goed beschermen tegen ziekteverwekkers van buitenaf (virussen, schimmels en bacteriën), zo ook tegen het ontwikkelen van goed- of kwaadaardige tumoren. Maar in sommige gevallen faalt het immuunsysteem en kunnen tumoren zich toch ontwikkelen. Standaard behandelingen voor vele vormen van kanker zijn: het operationeel verwijderen van tumoren, bestralen en/of chemotherapie. Hoewel deze behandelingen/therapieën succesvol kunnen zijn, kunnen ze ook schade aanrichten aan het gehele lichaam met matig tot ernstige bijwerkingen.

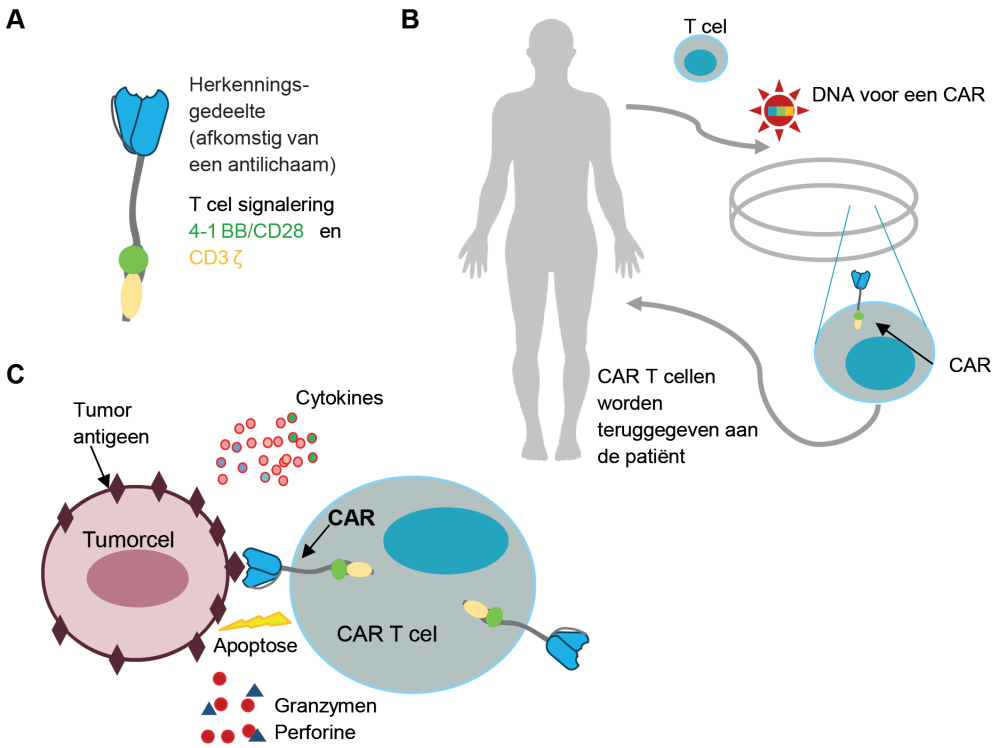
In de afgelopen decennia is daarom veel onderzoek gedaan naar het verbeteren van kankertherapie, door deze bijvoorbeeld specifieker te richten tegen de tumorcellen. Hierbij wordt gebruik gemaakt van het menselijke immuunsysteem en wordt 'immuuntherapie' genoemd. De afgelopen jaren heeft de ontwikkeling van immuuntherapie grote vooruitgang geboekt. Daarom is: 'Immuuntherapie voor de behandeling van kanker' in 2013 uitgeroepen tot doorbraak van het jaar door het wetenschappelijk tijdschrift *Science*[1]. Dit was deels te danken aan de succesvolle resultaten die behaald werden door behandelingen met T cellen die een specifieke receptor droegen: de chimere antigeen receptor (CAR)[2–4].

Normale T cellen hebben een sleutelrol bij de afweer zo ook tegen tumoren, maar om T cellen tegen een tumor te richten, moeten ze eerst een specifiek tumor-eiwit op de cel kunnen herkennen[5]. Dat doen ze normaliter via hun T cel receptor (TCR) die op het T cel oppervlakte aanwezig is. Echter, niet alle tumoren worden herkend door een TCR en dan kan er gebruik gemaakt worden van een CAR. Bij CAR T cellen vindt herkenning plaats via een slim samengestelde receptor die in het laboratorium is gemaakt op basis van een antilichaam, die via genetische manipulatie onderdeel is gemaakt van de T cel receptor[6,7]. Deze receptor heet dan een **c**himeer (bestaande uit 2 delen: antilichaam en TCR) en herkent een (tumor) molecuul op het celoppervlak (**a**ntigeen) met zijn **r**eceptor (CAR) (Figuur 1A en C). Een CAR gen kan met een speciale techniek (transductie), gebruikmakend van virussen[8], op de T cellen van een donor of patiënt worden gebracht. De kunstmatige receptor komt dan 'tot expressie' op T cellen. (Figuur 1B). Hierdoor, ontstaat er een sterke combinatie op de CAR T cel: één deel van de receptor voor herkenning door middel van het antilichaam gedeelte, een ander deel voor het signaleren tot het doden van het doelwit (de tumorcel) (Figuur 1C).

Het onderwerp van mijn onderzoeksproject is om een veilige en effectieve CAR T cel therapie voor multipel myeloom te ontwikkelen.

Multiple myeloom en CD38

Multiple myeloom (MM), ookwel ziekte van Kahler genoemd, is een vorm van beenmergkanker, dat veroorzaakt wordt door kwaadaardige witte bloedcellen (plasmacellen). Deze vorm van kanker beslaat 10% van alle bloed- en beenmergkankers



Figuur 1. (A) Schematisch overzicht van de chimere antigeen receptor structuur met herkenningsgedeelte afkomstig van een antilichaam en een of meerdere signaleringsdomeinen (4-1BB of CD28 en CD3ζ) (B) Toepassing van CAR T cel therapie, waarbij T cellen van een patiënt worden gehaald uit het bloed, in het laboratorium worden vervaardigd met een CAR en de tumor herkennende CAR T cellen worden teruggegeven aan de patiënt. (C) De CAR T cel doodt de tumorcel door apoptose te induceren en granzymen/perforine en cytokines uit te scheiden.

bij volwassenen wereldwijd. Patiënten hebben symptomen zoals botpijnen en –breuken, bloedarmoede, nierfalen en verminderde afweer. De afgelopen 30 jaar is de behandeling van MM sterk verbeterd, met hogere overlevingskansen[9]. Uit recent onderzoek, gedeeltelijk uitgevoerd door onze groep, is gebleken dat het antilichaam daratumumab zeer goed werkt bij MM patiënten[10–12]. Daratumumab is gericht tegen CD38, een molecuul wat in grote getalen aanwezig is (hoge expressie) op MM cellen.

In dit proefschrift bestuderen we de mogelijkheid om CAR's te richten tegen CD38. **Hoofdstuk 1** geeft een algemeen inleiding in CAR's, manieren om CAR therapie te verbeteren en de huidige CAR's die nu worden getest voor MM.

CD38-CAR T cellen

Omdat CD38 hoog tot expressie komt op MM cellen, hebben wij gebruik gemaakt van de herkenningsgedeelten van CD38 antilichamen en daarmee verschillende CD38-CAR's gemaakt, dit wordt beschreven in **hoofdstuk 2**. Gezonde T cellen van donoren werden

gebruikt om CD38-CAR's op te brengen. Deze CD38-CAR T cellen hebben we getest op beenmergmonsters van patiënten en bleken uiterst effectief om MM cellen te elimineren. Evenzo in een door ons ontwikkeld muizenmodel[13]. De behandelde muizen lieten inderdaad een sterk verminderde tumorgroei zien vergeleken met de controlegroep.

Echter, wat we ook zagen was dat CD38, hoewel in een veel mindere mate, ook voorkomt op gezonde cellen, voornamelijk op witte bloedcellen. Hoewel deze CD38-CAR T cellen zeer effectief leken tegen MM cellen, met hoge CD38 expressie, lieten ze ook een reactie zien tegen gezonde cellen met een lagere hoeveelheid CD38. Dit is uiteraard niet wenselijk, aangezien zulke CD38-CAR T cellen dan ook de gezonde cellen van patiënten zouden kunnen aanpakken.

Het specifieker maken door "fine tuning" van de affiniteit

Hoewel de gezonde witte bloedcellen ook CD38 hebben, is de hoeveelheid veel lager dan op MM cellen. Daarom wilden we een CD38-CAR maken die onderscheid zou kunnen maken tussen CD38-hoog versus CD38-laag. Daarvoor moet de CD38-CAR binding met CD38 enigszins verzwakt zijn, waardoor alleen binding kan optreden als er heel veel CD38 aanwezig is. Een dergelijke 'sterkte van binding' wordt ook wel affiniteit genoemd.

De eerste CD38-CAR's uit **hoofdstuk 2** waren geselecteerd op een sterke affiniteit voor CD38. In **hoofdstuk 3** gaan we daarom op zoek naar een geschikte CD38-CAR met een lagere affiniteit voor CD38. Zoals besproken in de inleiding bestaat een CAR uit een antilichaamgedeelte. In hoofdstuk 3 hebben we een hele reeks van nieuwe CD38 antilichamen getest op hun affiniteit met CD38. We vonden hiermee veel antilichamen die inderdaad CD38 herkende, met een 10 tot meer dan 1000 keer lagere affiniteit dan het oorspronkelijke sterkste antilichaam. Met geselecteerde antilichamen hebben we nieuwe lage affiniteit CD38-CAR T cellen gemaakt. De affiniteit van deze nieuwe CD38-CAR T cellen bleek, zoals verwacht, inderdaad onderscheid te maken tussen CD38-hoog op MM cellen en CD38-laag op gezonde cellen. In het MM muismodel, bleek er geen verschil tussen hoge en lage affiniteit CD38-CAR T cellen in de remming van tumorgroei. Maar, erg belangrijk, ook in een muismodel, lieten de lage affiniteit CD38-CAR T cellen geen negatief effect zien op gezonde witte bloedcellen, terwijl de hoge affiniteit dit wel deed. We waren dus in staat met een lagere affiniteit CD38-CAR het onderscheidend vermogen te verbeteren.

Effect van co-stimulatie

De lage affiniteit CAR T cellen die vergeleken werden in **hoofdstuk 3** hadden allen dezelfde T cel domeinen om de cel te activeren. Echter deze signaleringsdomeinen, kunnen veel invloed hebben op de T cellen en ze andere kenmerken geven. CAR's zijn samengesteld uit een antilichaam en een gedeelte van de T cel receptor (TCR), het CD3ζ domein (Figuur 1A). Er is veel onderzoek gedaan naar het toevoegen van 'co-stimulatie' aan CAR's. Co-stimulatie, zoals de naam suggereert, geeft dus naast de gewone stimulatie/activatie

(via CD3) een extra stimulus. Er bestaan veel smaken van deze co-stimulatie, de meest bestudeerde zijn de domeinen: 4-1BB en CD28. Grofweg geeft 4-1BB het signaal om T cellen te laten delen (prolifereren), terwijl CD28 veel dodelijker is voor de tumor cel[14,15]. In hoofdstuk 2 en 3 waren allen CAR's gemaakt met een 4-1BB signaal. In **hoofdstuk 4** zijn we daarom geïnteresseerd in de vervanging van dit gedeelte voor een CD28 gedeelte en ook wanneer de cellen beide signalen kunnen ontvangen, dus CD28 en 4-1BB. Wat meteen opviel was dat de lage affiniteit CAR's die we in hoofdstuk 3 hadden gekozen ineens veel sterker bleken te zijn wanneer de co-stimulatie naar CD28 veranderd werd. Om gezonde cellen te beschermen, ging de keuze nu uit naar een CAR met een *nog* lagere affiniteit.

Hoewel CD28 voor sterkere CAR's zorgde, bleek de toevoeging van 4-1BB signalering de CAR's ook positief te beïnvloeden omdat ze beter konden prolifereren en minder snel uitgeput raakten. Ook in de muizen bleven de CAR T cellen beide co-stimulaties (CD28+4-1BB) ook langer in de muizen aanwezig[14]. De overgebleven aanwezige T cellen onderdrukten nog steeds de tumorgroei. De lage affiniteit van de CD38-CAR T cellen beschermde in alle gevallen nog steeds de gezonde cellen. Het type co-stimulatie heeft dus een sterke invloed heeft op prestaties van een lage affiniteit CAR.

Veiligheid van een CAR

Naast het verlagen van de affiniteit uit **hoofdstuk 3 en 4** kunnen we het effect van CAR T cellen ook beïnvloeden door de CAR expressie aan en uit te schakelen.

In **hoofdstuk 5** beschrijven we de mogelijkheid om met een antibioticum de genexpressie, het afschrijven van de DNA code voor een CAR, te reguleren. In de klinische praktijk betekent dit dat de CAR pas op de T cel wordt gebracht wanneer de patiënt met een medicijn (een antibioticum) wordt behandeld. Door te stoppen met toediening, verdwijnt de CAR weer en is het mogelijke 'gevaar' van de CAR geweken[20]. In onze experimenten zagen we inderdaad dat binnen enkele uren de expressie van de CAR te meten was en het expressieniveau ook lager bleek bij een lagere dosering van het antibioticum. Door het antibioticum te verwijderen, door de cellen te wassen, nam expressie langzaam af. Ook hierin zagen we dat de cellen met een lage expressie een voorkeur hadden voor de MM cellen en niet de gezonde cellen. Dit suggereert dat zowel de lagere affiniteit, maar ook de gereguleerde expressieniveaus, een mogelijkheid bieden om eventuele nadelige effecten van CAR T cellen te verminderen.

Een universele CAR

De huidige manier van CAR T cel therapie is weergegeven in figuur 1, cellen worden van de patiënt afgenomen en na het implementeren van de CAR weer teruggegeven. Dit is over het algemeen een arbeidsintensieve, dure, maar ook veilige manier van toedienen. Om CAR therapie breder toe te passen kunnen mogelijk andere cellen een oplossing bieden. T cellen hebben de goede eigenschap kleine cel verschillen te herkennen, maar vergroten daarmee ook de kans op graft-versus-hostziekte, wat betekent dat T cellen van

een donor een patiënt kunnen aanvallen. Een mogelijkheid om dit te voorkomen is het gebruiken van andere cellen, bijvoorbeeld natural killer (NK) cellen.

In **hoofdstuk 6** hebben wij gekeken naar een aparte kleine groep binnen de witte bloedcellen: de invariante natural killer T (iNKT) cellen. Deze groep cellen deelt eigenschappen van zowel NK cellen als T cellen[16]. Ze hebben een snelle 'killer'functie, maar ook een T cel receptor, net als T cellen. In de 'invariante' NKT cellen, zijn alle T cel receptoren gelijk en herkennen allemaal kleine structuren (suikerachtige lipiden) in het molecuul CD1d[17]. Tumorcellen zoals MM kunnen in CD1d een lipide laten zien die T cellen activeren[18]. Als we in de iNKT cellen een CD38-CAR introduceren kunnen deze cellen dus twee dingen herkennen: lipiden in CD1d en CD38. Hierdoor kunnen we gebruik maken van beide receptoren om de iNKT cellen te laten prolifereren en tumor cellen te doden. Daarnaast vormen iNKT cellen, door hun CD1d-restrictie in tegenstelling tot T cellen, geen allo-reactieve respons en beschermen zelfs tegen graft-versus-hostziekte[19]. Met deze strategie is het dan mogelijk om een grote patiëntenpopulatie met hetzelfde celproduct te behandelen.

Conclusie

Immuuntherapie is een veelbelovende vorm van kankertherapie. Klinische studies met CAR T cellen hebben laten zien hoe sterk dit anti-tumorwapen is. Om CAR T-celtherapie voor meer patiënten toegankelijk te maken, wordt er gewerkt aan verbetering en om toxiciteit te verminderen. Deze belangrijke aspecten van de ontwikkeling van CAR T-cellen zijn bestudeerd en gepresenteerd in dit proefschrift: naar een veilige en toepasbare CAR T-celbehandeling voor multipel myeloom. In **hoofdstuk 7** worden de resultaten uit dit proefschrift samengevat en bediscussieerd en worden suggesties gedaan voor toekomstig onderzoek.

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APPENDIX

CURRICULUM VITAE

LIST OF PUBLICATIONS

LIST OF CONFERENCE PRESENTATIONS

DANKWOORD/ ACKNOWLEDGEMENTS

CURRICULUM VITAE

Esther Drent werd geboren op 14 maart 1989 te Wijk bij Duurstede. In 2007 behaalde zij haar Atheneum diploma aan het Revius Lyceum te Doorn. In hetzelfde jaar startte zij haar bachelor biomedische wetenschappen gevolgd door de master Infection & Immunity aan de Universiteit Utrecht. De eerste experimenten met het transduceren van T cellen werden gedaan als onderzoeksstage in UMC Utrecht, in de groep van Prof.dr. J. Kuball. Verdere onderzoekservaring deed zij op tijdens een buitenlandse masterstage in de groep van Prof.dr A. Garcia-Sastre aan de 'Icahn School of Medicine at Mount Sinai' in New York, VS. In 2012 behaalde zij haar masterdiploma en startte daarna met haar promotieonderzoek in het UMC Utrecht onder supervisie van Prof.dr H.M. Lokhorst, dr. N.W.C.J. van de Donk en dr. T. Mutis. In 2014 verhuisde deze groep naar het Cancer Center Amsterdam in het VUmc en werd haar promotieonderzoek hier verder doorgezet. In 2018 startte Esther haar onderzoek als junior scientist in het research & development bedrijf, Gadeta, gefocust op 'T cel therapie' in het Utrecht Science Park.

Esther Drent was born 14 March 1989 in Wijk bij Duurstede. In 2007 she completed her secondary education (atheneum) at the Revius Lyceum in Doorn. In the same year she started her bachelor biomedical sciences, followed by the master Infection and Immunity at the Utrecht University. The first experiments, as part of her first internship, started with transducing T cells at the UMC Utrecht in the group of Prof.dr. J. Kuball. She obtained more research experience during her master-internship abroad, in the group of Prof.dr. A. Garcia-Sastre at the 'Icahn School of Medicine at Mount Sinai' in New York, USA. In 2012 she graduated for her masters and started her research underlying this thesis at the UMC Utrecht, under the supervision of prof.dr. H.M. Lokhorst, dr. N.W.C.J. van de Donk and dr. T. Mutis. In 2014 this group moved to the Cancer Center Amsterdam at the VU university medical center, where her research was carried out further. In 2018 Esther started as a junior scientist in the research & development company, Gadeta BV, focusing on 'T cell therapy' in Utrecht Science Park.

LIST OF PUBLICATIONS

Drent E, Groen RW, Noort WA, Themeli M, Lammerts van Bueren JJ, Parren PW, Kuball J, Sebestyen Z, Yuan H, de Bruijn J, van de Donk NW, Martens AC, Lokhorst HM, Mutis T. Pre-clinical evaluation of CD38 chimeric antigen receptor engineered T cells for the treatment of multiple myeloma. *Haematologica. 2016 May;101(5):616-25. doi: 10.3324/haematol.2015.137620*

Drent E, Themeli M, Poels R, de Jong-Korlaar R, Yuan H, de Bruijn J, Martens ACM, Zweegman S, van de Donk NWCJ, Groen RWJ, Lokhorst HM, Mutis T. A Rational Strategy for Reducing On-Target Off-Tumor Effects of CD38-Chimeric Antigen Receptors by Affinity Optimization. *Mol Ther. 2017 May 13. doi: 10.1016/j.ymthe.2017.04.024.*

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Drent E, Poels R, van de Donk NWCJ, Zweegman S, Lokhorst HM, Groen RWJ, Mutis T, Themeli M. Co-stimulatory domains can restore the reduced functionality of lower affinity CARs. Selected as **oral presentation** at the Dutch Hematology Congress, 24-26 January 2018, Papendal, The Netherlands.

Drent E, Poels R, van de Donk NWCJ, Zweegman S, Lokhorst HM, Groen RWJ, Mutis T, Themeli M. The combination of 41BB and CD28 costimulatory signals endows lower affinity CAR T cells with the most optimal properties for selective targeting of the Tumor Associated Antigen CD38. Selected as **poster presentation** at the 59th ASH annual meeting 9-12 December 2017, Atlanta, USA.

Drent E, Themeli M, Poels R, van de Donk NWCJ, Zweegman S, Groen RWJ, Lokhorst HM, Mutis T. A rational strategy for reducing on-target off-tumor effects of CD38-Chimeric Antigen Receptors by affinity optimization. Selected as **oral presentation** at the Cellular therapy, 16-17 March 2017, Erlangen, Germany.

Drent E, Themeli M, Poels R, van de Donk NWCJ, Zweegman S, Groen RWJ, Lokhorst HM, Mutis T. Reducing on-Target Off-Tumor Effects of CD38-Chimeric Antigen Receptors By Affinity Optimization. Selected as **oral presentation** at the 11th Dutch Hematology Congress, 25-27 January 2017, Papendal, The Netherlands.

Drent E, Themeli M, Poels R, van de Donk NWCJ, Zweegman S, Groen RWJ, Lokhorst HM, Mutis T. Reducing on-Target off-Tumor effects of CD38-Chimeric Antigen Receptors by Affinity Optimization. Selected as **poster presentation** at the 58th ASH annual meeting 3-6 December 2016, San Diego, USA.

Drent E, Themeli M, Lammerts van Bueren JJ, Rademaker R, Riedl T, Verzijl D, Parren PWHI, van de Donk NWCJ, Lokhorst HM, Mutis T. Screening for affinity divergent CD38 antibodies and their translation into CD38 Chimeric antigen receptors. Selected as **oral presentation** at the Dutch Tumor Immunology Meeting, 9-10 June 2016 Breukelen, The Netherlands.

Drent E, Themeli M, Lammerts van Bueren JJ, Rademaker R, Riedl T, Verzijl D, Parren PWHI, van de Donk NWCJ, Lokhorst HM, Mutis T. Optimizing the affinity of CD38 Chimeric Antigen Receptor (CAR) T cells to improve their specificity for Multiple Myeloma cells. Selected as **oral presentation** at the 10th Dutch Hematology Congress, 20-22 January 2016, Papendal, The Netherlands

Drent E, Lammerts van Bueren JJ, Parren PWHIP, Kuball J, Sebestyen Z, van de Donk NWCJ, Noort W, Groen R, Martens A, Lokhorst HM, Mutis T. CD38 Chimeric Antigen Receptor Engineered T cells as Therapeutic Tools for Multiple Myeloma. Selected as **poster presentation** at the Society for Immunotherapy of cancer (SITC), 4-8 November 2015, National Harbor, USA.

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Drent E, Lammerts van Bueren JJ, Parren PWHI, Kuball J, Sebestyen Z, van de Donk NWCJ, Noort W, Groen R, Martens A, Lokhorst HM, Mutis T. CD38 Chimeric Antigen Receptor engineered T cells as tools for immunotherapy of Multiple Myeloma. Selected as **poster presentation** at the 56th ASH annual meeting 6-9 December 2014, San Francisco, USA.

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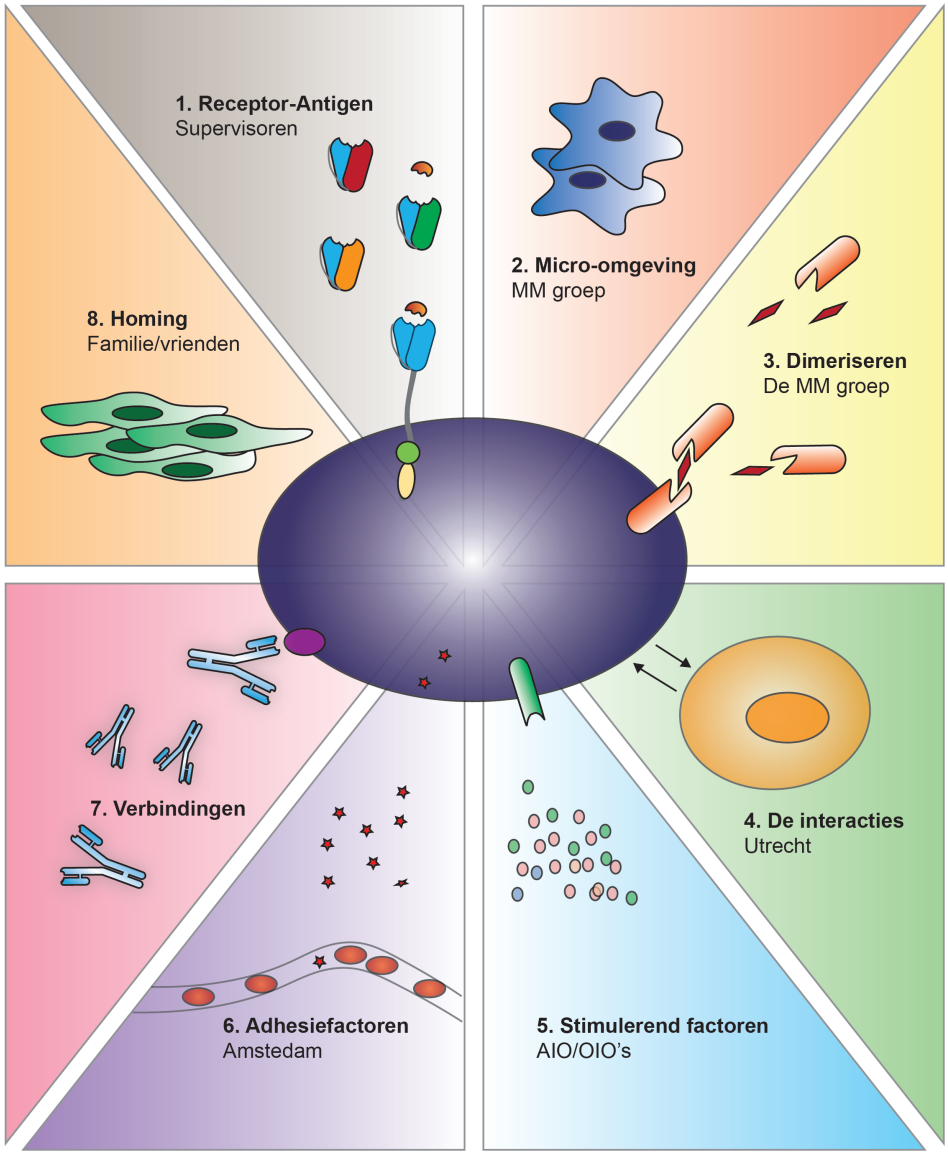
Drent E, Lammerts van Bueren JJ, Parren PWHI, van de Donk NWCJ, Lokhorst HM, Mutis T. CD38 CARs as a potential treatment for Multiple Myeloma patients. Selected as **oral presentation** at the Dutch Tumor Immunology Meeting, 5-6 June 2014 Breukelen, The Netherlands.

Drent E, Lammerts van Bueren JJ, Parren PWHI, van de Donk NWCJ, Lokhorst HM, Mutis T. CD38 CARs as a potential treatment for Multiple Myeloma patients. Selected as **oral presentation** at the 8th Dutch Hematology Congress, 22-24 January 2014, Papendal, The Netherlands

DANKWOORD/ACKNOWLEDGEMENTS



Bij het werken aan een PhD project, moest ik soms denken aan schematische weergaven van allerlei immunologische processen die bijdragen aan het effect van een cel. Zoals aangegeven op het figuur bij dit dankwoord. Zo zijn er factoren die kunnen stimuleren of inhiberen, bindingen aangaan of blokkeren en bevindt de cel zich in een bepaalde micro-omgeving. Dit alles draagt bij aan een goed werkende cel. Dit is een vergelijking met hoeveel mensen (supervisoren, groepsleden, postdocs, PhD-studenten, analisten, samenwerkingen, vrienden en familie), invloed op mij of mijn projecten hebben gehad. Alle factoren bij elkaar hebben bijgedragen tot dit proefschrift. Bedankt!

1. De receptor + antigen de supervisors

Signaal 1, de activatie doordat een antigen een receptor bindt. Daarvoor wil ik **Tuna**, mijn mentor, bedanken! We hebben veel bereikt en meegemaakt in 5 jaar. Bij mijn vertrek heb ik niet voor niets een Tuna manual geschreven voor allerlei mensen na mij, want je hebt af en toe wel een handleiding nodig. Maar ook om te laten zien hoe goed je elkaar leert kennen en keer op keer weer iets moet/wil neerzetten samen. Dankzij jou heb ik kritisch leren denken, jouw passie voor wetenschap is enorm en jij durft altijd die ene lastige vraag te stellen. Je kunt outside-the-box denken en daar heb ik veel van geleerd. Als ik jou met data kon overtuigen dan pas was het een goed experiment. Bedankt!

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&

2. De Micro-omgeving: de MM groep supervisors

Daarnaast zijn er nog meer staffleden/supervisors van de MM groep, die zorgen voor een goede bodem, of een micro-omgeving. **Anton**, bedankt voor alle input over de muisexperimenten en de groepsuitjes in jouw achtertuin en kano-avonturen. **Richard**, geweldig hoe jij en Anton alle (muis)experimenten hebben overgezet van Utrecht naar Amsterdam. Volgens mij loopt alles nu erg soepel, bedankt voor je hulp en adviezen met zowel *in vitro* en *in vivo* werk!

Maria, in het Nederlands oké? Je was een belangrijke supervisor voor mij! Je gedrevenheid en passie zijn bewonderenswaardig. Met jouw komst is het CAR werk enorme gegroeid, bedankt voor je kritische kijk en ervaring met al onze CAR's. Succes, je bent straks de perfecte combinatie tussen diehard onderzoeker en arts!

3. Dimeriseren: de MM groep

Dimeriseren, de researchgroep die werkt aan hetzelfde onderwerp, zorgt ervoor dat we samenkomen. Dank aan de hele MM groep die als team enorm gegroeid is en veel veranderingen heeft meegemaakt. Steeds een andere samenstelling, maar een dynamische, flexibele gemotiveerde groep mensen!

Maar als belangrijkste: **Renée.** Rots in de branding! Jij kwam vers van de HLO en hebt alles van begin tot eind geleerd of zelfs zèlf ontwikkeld. Als echt team hebben we veel samen gewerkt, en uiteindelijk maakten we elkaars zinnen af. Ik vertrouw je met alles en had het niet zonder je gekund. Ik gun iedereen een topanalist als Renée. Je nieuwsgierigheid en

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4. De interacties: UMC Utrecht

Maar... het begon allemaal in Utrecht, de stad waar ik bijna 10 jaar woonde en weer naar terug keer. Daar begonnen de interacties en heb ik veel geleerd van o.a. Tineke, Ruud, Maarten en Berris. **Tineke**, het voelde alsof je de moeder was op het lab, bedankt voor je rust, vertrouwen en adviezen. **Ruud en Maarten**, jullie waren een komisch duo (met flauwe grappen) op het kweeklab! Maarten, extra dank voor het opzetten en helpen met alle (facs)experimenten in het CCA! **Berris**, wat was ik blij dat jouw humor, vastberadenheid en ervaringen meegingen naar Amsterdam! Wie weet zien we elkaar nu op de Uithof.

Bijna als grote zussen wisten **Rimke, Sanne en Inger**, me te helpen met het opstarten van mijn onderzoek. **Rimke**, altijd vrolijk en jouw positieve en nuchtere kijk op dingen, vond ik altijd prettig en stelde me gerust. **Sanne**, wat was het gezellig om zowel experimenten als badkamerindeling met je uit te denken. Ook je kritische blik op onderzoek en '3-uit-de-5 praatjes' tactiek pas ik nog steeds toe. **Inger**, dank voor je hulp, ook met de laatste PhD zaken. Jouw passie voor het klinische maar ook translationele onderzoek ga je fantastisch doorzetten in het VUmc. Dan was er **Jonas**, jouw grappige adviezen hielpen me goed op weg, of verwarde me soms, haha! **Laurens**, van begin tot het eind! We zijn samen begonnen, jammer dat het einde moest in jouw afwezigheid in het CCA. Maar altijd veel bij te praten en te lachen onder het genot van een kopje koffie. Bedankt voor de mooi tijd en succes met alles en plezier weer terug in het VUmc. **Jakub**, lunch buddy, your smile when I came to pick you up for lunch, I'll never forget. Our weird conversation in a mixture of Dutch, English, Danish and Czech, no idea about what, but it was great! **July**, we only worked together for a short while, loved our chats! Verder wil ik graag **Cor en Simone** bedanken op het lab. En de **Lotte S, Lotte W, Marjolein**, met wie ik korte tijd op

de LTI AIO kamer heb gezeten! Verder was de UU Graduate School of Life Sciences een gezellige groep met PhD retreats en borrels!

5. De stimulerende factoren: the PhD students CCA

The large number of PhD students that I've met, were in many ways 'stimulating factors'. Even if we do not directly work together, we help each other out and laugh and sometimes cry together. You guys we're not only 'stimulating', but also nurturing and motivating! Toen we in het CCA kwamen waren daar **Eline, Wendelien, Denise**, de dames achter het schot, bedankt voor jullie wegwijs en warme welkom in de AIO kamer. **Nathalie**, Utrecht-trein buddy en altijd wat te kletsen, jouw huis-, trouw- en reisplannen zijn nog steeds een inspiratie voor me. **Anna**, you're so funny and you were the wisest in the room. I loved our chats over a coffee and shared sense of humor. **Carolien A**, je uitspraken zijn nog steeds hilarisch en komen soms nog steeds naar boven, thanks! **Han**, jouw gedrevenheid werkte motiverend! En je (lichte) obsessie voor IGFBP7 (gezien je IGFP007 pc bureaublad), succes met afronden! **Rocco**, while staring into the distance in the AIO room, I was often by accident staring at you, haha. Our talks, happy or sad, were always very good and got us both further, thank you! **Lisa**, in alle dimensies steven jij af op het ontrafelen van de stromale interacties. Erg gezellig om met je te werken en experimenten te bedenken. **Margot**, bedankt voor je soms aanstekelijke enthousiaste vibe en bevlogenheid in het onderzoek. **Diana**, de sort specialist, bedankt voor de leuke enerverende gesprekken op de AIO kamer. Lieve **Hilma**, wat hebben we gelachen, de 'senior' en de 'junior' in Atlanta en New York. Als ik moest kiezen met wie ik op schiphol vast moet zitten, dan zou ik wederom jou kiezen. **Noortje**, of soms evil Noortje! Bedankt voor alle koekjes, boodschappen en top AIO-weekend. **David**, haha jouw heerlijke quotes en humor, maar ook vastberaden (tellen, tellen, tellen) in het lab! **Inge**, buurvrouw, je bent een splice-queen to be, bedankt en succes! **Kris**, arts-onderzoeker in de dop, wat heb je al een hoop bergen verzet, bedankt ga zo door, succes!

Jort, succes met je geduelleer met de CARs, bedankt voor de leuke samenwerking! **Carolien D** met al je wilde flow plannen al algoritmes, zet 'm op! Jij moet nu iedereen meenemen om 12 uur. **Afroditi**, you're doing great, give everyone some greek vibes! Your wedding was amazing, with special thanks to the greek-group. Let's repeat this year? **Ittai,** jammer dat je ergens anders zit, maar borrels en het AIO-weekend met jou erbij maakten het extra leuk! **Wang,** good luck with your start at the CCA and living in the Netherlands.

Daarnaast wil ik graag mijn studenten **Lisette, Manon en Cilia**, bedanken voor hun inzet en wat ik ook van hen heb geleerd. In het bijzonder **Manon**, na een turbulente verhuizing, werden we ook wel jut en jul genoemd toen we door het CCA aan het zoeken waren. Bedankt voor je hulp en positieve kijk op alles!

6. De adhesie factoren: VUmc CCA

Allereest dank aan iedereen in het CCA die de tijd en ruimte hebben gegeven om de 'Utrecht-groep' te helpen integreren in het VUmc/CCA.

Sonja, jouw warme welkom en mooie speeches deden me altijd erg goed, bedankt voor alle positieve energie, vertrouwen en interesse. **Gert, Arjan, Martine, Marielle, Jeroen en José** bedankt voor alle tips en adviezen tijdens de researchbesprekingen. **Gerrit-Jan, Linda** en **Jacqueline**, bedankt voor het sturen en stimuleren van het onderzoek in het CCA en de tips en scherpe blik tijdens besprekingen.

Sander, ik wist je altijd te vinden met die ^#&@* gloeiende, plaatreader. Bedankt voor je flauwe grapjes en ook oplossingen! Daarbij ook **Jennifer**, bedankt voor FACS/BD-bel hulp en gezelligheid tijdens de lunch. **Fedor**, als ML2 LaMa, bedankt voor je hulp en quality checks. **Adri**, onze brandblusser, bedankt voor grappige luchtige kijk op alles. **Floortje**, bedankt voor de hulp met opstarten en de gesprekken in het kweeklab. **Marisa** bedankt voor je input in het (FACS)onderzoek. We zijn met zo'n grote afdeling, maar iedereen heeft op zijn/haar manier bijgedragen aan het onderzoek en leuke tijd in het CCA. **Tania, Costa, Angèle, Paulien, Johan, Bianca, Zinia, Payam, Maike, Maaike, Adrian, Eline, Willemijn, Karlijn, Vivianne, Dennis, Anne, Anouk, Yvonne V, Yvonne O, Claudia, Jannemieke, Arjo en Jan-Willem** bedankt voor hulp en gezelligheid op het lab!

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7. De verbindingen/the connections

Verbindingen zijn er veel geweest, in de vorm van samenwerkingen of gedachtewisselingen. Bedankt aan alle groepen met wie we samengewerkt hebben. Een jaar lang heb ik 1 dag in de week bij Genmab gewerkt. Toen dit tegelijk plaatsvond met de verhuizing naar Amsterdam, was het soms even schipperen. Vooral **Jeroen Lammerts van Bueren**, bedankt voor de besprekingen, alle ideeën en het uiteindelijk doorzetten van de low-affinity CARs. Daarbij ook **Rik Rademakers, Dennis Verzijl** en **Thilo Riedl** en iedereen bij Genmab bedankt voor het meedenken, uitvoeren en analyseren van de experimenten!

Daarnaast ook de verbindingen tussen UMC's. Dank aan **Cordula, Zsolt, Wouter** en **Trudy**, in de Kuball group, voor hulp met het opstarten van de kloneringen in het UMCU.

Daarnaast ook zelfs verbindingen met andere landen. Research can make a lot of connections, thanks to Michel Sadelain and the **Sadelain group**, thanks to Maria we join efforts and share ideas.

And of course special thanks to the **committee**, Tanja de Gruijl, Marie-José Kersten, Mirjam Heemskerk, Jürgen Kuball & Michel Sadelain. Thank you for carefully reading my thesis. In addition also Linde Meyaard and Paul Parren, I am looking forward to our discussions during the defense!

8. Homing

De homing is waar de cel naar toe gaat en zich op het beste voelt. Voor mij is dat bij vrienden en familie.

Lieve vrienden van VLF: **Andrea & Michiel, Anna & Luc, Anneloes & Bert, Bram, Eline & Jasper, Karin, Kjartan & Tonca, Leonie & Ruud, Sven & Amber!** Als we bij elkaar zijn herinnert het aan een top studententijd en ieder jaar eindigt en begint goed met deze groep! Dames van VLF, A, A, A, K, L, jullie zijn in het bijzonder een steun en vraagbaken voor mij, thanks!

BMW chickies: **Anne, Janneke, Judith, Erinke, Kirsten en (nog een keer) Eline**, we gingen tegelijk naar het einde van de studie en wat delen we altijd veel (PhD) ervaringen, overwegingen en (meiden)gezelligheid, dankjulliewel!

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