Activation-inducible CAR expression enables precise control over engineered CAR T cell function

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Abstract

CAR T cell therapy is a rapidly growing area of oncological treatments having a potential of becoming standard care for multiple indications. Coincidently, CRISPR/Cas gene-editing technology is entering next-generation CAR T cell product manufacturing with the promise of more precise and more controllable cell modification methodology. The intersection of these medical and molecular advancements creates an opportunity for completely new ways of designing engineered cells to help overcome current limitations of cell therapy. In this manuscript we present proof-of-concept data for a novel engineered feedback loop. We manufactured activation-inducible CAR T cells with the help of CRISPR-mediated targeted integration. This new type of engineered T cells expresses the CAR gene dependent on their activation status. This artifice opens new possibilities to regulate CAR T cell function both in vitro and in vivo. We believe that such a physiological control system can be a powerful addition to the currently available toolbox of next-generation CAR constructs.

Introduction

Genetically engineered T cells expressing modified T Cell Receptor (TCR) or Chimeric Antigen Receptor (CAR) are powerful tools to treat tumor malignancies (Kochenderfer et al 2010). Until today, several CAR T cells therapies against hematological disorders have been approved with plethora of others being evaluated in clinical trials (Adami & Maher 2021). All the current commercial products use viral vector-based gene delivery (MacKay et al 2020). Although this is a well-established and relatively safe strategy, it possesses some significant limitations. Mechanistically, contemporary viral vectors rely on uncontrolled semi-random integration. Although safety of viral vectors is proven by the increasing number of patients treated with CAR T cell products and safety viral gene delivery has been accepted by health agencies, the inherent risk of insertional mutagenesis remains an unsolved issue. (Fraietta et al., 2018; Ruella et al., 2018; Bishop et al., 2021). Therefore, next-generation T cell manufacturing aims to use better controlled gene editing methods such as CRISPR/Cas. CRISPR/Cas enables precise targeted gene integration into pre-defined location within the genome (Jinek et al., 2012). This in turn not only makes the editing procedure more precise but also opens an opportunity to take advantage of the gene integration site.

To date all clinically used CAR gene cassettes are driven by strong, constitutively active artificial promoters, overwriting the dynamic transcriptional regulation that occurs in a physiological setting with direct impact on activation kinetics, phenotype, and survival. Continuous CAR expression can lead to exhaustion and suboptimal CAR functionality in vivo (Frigault et al., 2015). The CRISPR/Cas technology allows gene transfer downstream of any endogenous promoter, thus reducing the need of use of engineered or external promoters and focus on more physiologic cell modifications (Roth et al., 2018; Schober et al., 2019). Native promoter regions enable highly regulated gene transcription. The complex sequence patterns funnel regulatory cues from distal enhancers and their associated regulatory proteins leading to distinct transcription (Haberle & Stark 2018). Preserving the sophisticated physiological gene regulation may help to engineer superior cell products. It has been demonstrated that better targeting of CARs and engineered TCRs brings clear benefits to the potency and function of edited T cells by inserting artificial genes under control of endogenous TCR promoters thus providing a more physiological (trans-)gene expression (Eyquem et al. 2017).

Moreover, a well-designed editing strategy can enhance precision of CAR-mediated killing. CAR T cells are very potent in the lysis of cells expressing target antigen (e.g., CD19 or BCMA), but they do not distinguish between healthy and abnormal cells (Maude et al. 2018, Killock 2020, Munshi et al 2021). Thus, after performing their initial task of tumor clearance, CAR T cells continue to attack all cells expressing their cognate antigen (Kalos et al. 2011). Several approaches to circumvent these on-target off-tumor side effects of persisting CAR T cells have been proposed. For example, CAR T cells can be eradicated after complete tumor clearance by systemic administration of antibodies directed against a synthetic "kill switch" co-expressed on CAR T cells, leading to complete and persistent B cell recovery (Paszkiewicz et al 2016). Another option is mRNA-based transient expression of CAR, which re-directs T cells against target cells as long as mRNA is systemically supplied (Juillerat et al

2020, Rurik et al 2022). However, such approaches have challenges of addressing distribution kinetics, precision, penetrance, and persistence of applied molecules.

Another method of reducing damage to healthy tissue is logically gated CAR T cells (Roybal et al 2016). For example, 'AND' gated CAR T cells can differentiate tumor cells from healthy tissue more precisely since they only execute effector function by dual antigen recognition on target cells (Srivastava et al 2019). However, healthy tissue destruction is only securely prevented if both antigens are spatially separated enough to prevent dual recognition. In addition, 'NOT' and 'OR' gated CARs were also described in preclinical models (Han et al 2019). These logic gates seem to be a very appealing and sophisticated approach to regulate edited T cell function but require complex cell engineering and the genetic payloads are bulky (Kloss et al 2013).

In this article, we propose an alternative and novel approach to solve the issue of persistent on-target off-tumor tissue destruction after complete tumor clearance using control over TCR/CAR gene expression. Our strategy aims at reducing necessary genetic cargo and relies instead on physiological regulation of the engineered protein. To this end, we propose the use of constructs which are embedded at loci of molecules that are temporarily expressed via physiological signal cascades. In case of T cells, apparent target loci are downstream of promoters that upregulate their cognate proteins upon T cell activation (e.g.: Nur77, FoxP3, CD69, PD-1, HLA-DR) (Conley et al 2016, Gros et al 2014). With this approach we created a temporal regulatory system where persistence of T cell stimulation (exogenous or endogenous) is prerequisite to exert CAR/TCR function. We call it an engineered feedback loop: when cells are active, target construct is expressed. In this study, we demonstrated feasibility through in vitro and in vivo proof-of-concept experiments. We are convinced that our engineered feedback loop can be utilized in advanced cell therapy applications.

Results

We conceptualized that any theoretical construct can be regulated in a similar fashion to endogenous up or downregulation if it is targeted into an appropriate locus and inserted via homologous recombination. As a proof-of-concept example, we selected anti-CD19 CAR T cells as a relevant setting for therapeutic T cell engineering (Figure 1A). In this case, the CAR construct is integrated into the area downstream of a T cell activation-dependent promoter, simultaneously disrupting the coding sequence of an endogenously coded protein. Of note, for intended evaluations, TCR/CD3 complex was left unedited, enabling classical anti-CD3/CD28 T cell stimulation that is independent of anti-CD19 antigen-specific activation. In our example, polyclonally stimulated T cells are modified with the CRISPR/Cas9 system, whereby the CAR gene is integrated into the specific gene locus substituting the downstream sequence with the CAR (Figure 1A). As long as cell activation is maintained (especially in an antigen-specific setting) the CAR continues to be expressed creating a positive feedback loop (activation via CAR target recognition facilitates further CAR protein production) (Figure 1A). This engineered feedback loop allows control of CAR expression by the cell activation state.

To test our hypothesis, we evaluated several well-known T cell molecules that are upregulated upon stimulation (Figure 1B). As selection criteria, we assessed expression kinetics, the restimulation potential as well as biological significance. We pre-selected two classical T cell activation markers – CD25 and CD69 – but tested upregulation of PD-1 as well. The PD-1 receptor is not only upregulated upon T cell stimulation, but a prolonged presence of this marker is correlated with T cell exhaustion (Gros et al 2014). Therefore, disruption of PD-1 can provide significant benefits for CAR T cell persistence (Wang et al 2021). Hence, for first experiments we focused on CAR targeting into the PD-1 locus in order to establish a 2-in-1 model, in which we combine engineered feedback loop CAR expression with the benefit of PD-1 knock-out (KO). In addition, to demonstrate broader application of our concept we also tested targeting of CD69 locus. CD25 turned out to be unsuitable for our approach since its expression was elevated even throughout resting phase (Figure 1B, Supplementary Figure 1). In our model we used human primary T cells stimulated with soluble anti-CD3/CD28 reagent (Expamers) and used a non-viral CRISPR/Cas9 delivery system and dsDNA HDR template as previously described (Poltorak et al., 2020; Schober et al., 2019). Edited T cells readily expressed the CAR construct but had almost no residual expression of PD-1, indicating successful targeted integration as well as very efficient PD-1 KO (Figure 1C, Supplementary Figure 2). These cells were then either left unstimulated to reach a resting phase or restimulated with an additional dose of Expamers. 24 h post-restimulation, CAR expression was evaluated (Figure 1D). Conversely to the controls (CAR gene knock-in (KI) into TRAC locus and CAR randomly integrated by LV transduction), expression of CAR construct located downstream of the PD-1 promoter significantly increased after restimulation (Figure 1D). This was a first experimental validation that engineered feedback loop CAR expression can be achieved through our approach. Interestingly, CAR expression in CRISPR/Cas edited cells seems to increase in vitro spontaneously over time (and irrespectively of activation state) in contrast to LV-mediated randomly integrated receptors (Figure 1D) again pointing to the incomplete

PD-1 downregulation in resting phase in this in vitro setting and might hint to an improved fitness of CAR engineered T cells (1B).

Next, we decided to test the robustness of our inducible CAR concept over an extended period of two rounds of stimulation (Figure 1E). For this purpose, we expanded CAR T cells using B-cell-derived Lymphoblastoid Cell Line (LCL), which presents CD19 target antigen. After one week of co-culture, we observed significant cell expansion in all samples of interest indicating productive antigen-specific cell responses. Interestingly, fold-increase in cell expansion was higher in T cells with CAR controlled by the PD-1 promoter compared to the LV control (Figure 1E). To further examine maintenance of the initially reported behavior of engineered feedback loop CAR T cells and to improve cellular resolution of the assays, we assessed the response of LCL-expanded cells under polyclonal and antigen-specific restimulation (Figure 1F). In particular, we evaluated whether dynamic upregulation of CD25 and CD69 activation markers matches anticipated kinetic of engineered feedback loop CAR expression. As expected, after polyclonal restimulation both CD25/CD69 and CAR content increased significantly at day 2 post restimulation and returned to baseline levels at day 6 (Figure 1F, blue line). Noteworthy, only in the group of activation-inducible CAR T cells increase in activation markers correlated with transient rise of CAR expression (blue line). This contrasted with the antigen-specific restimulation, where enhanced activation corresponded to higher CAR content indicating expansion of CAR+ cells upon target engagement irrespectively of CAR integration strategy whereby activation markers are upregulated until d6 reaching baseline later on d9 after complete target cell elimination (Figure 1F, red line). These data confirmed that proposed engineered feedback loop does not diminish over time and becomes a permanent feature of edited cells. Of note, during all our studies we observed heightened baseline level of CAR (approx. 40%) (Figure 1F) over steady state PD-1 presence (<20%) Figure 1B). This may be the outcome of editing strategy and an impact of PD-1 KO on general T cell biology or an artefact of an in vitro culture.

After verifying activation-inducible CAR expression in practical experiment, we decided to test the functionality of generated CAR T cells and potential benefits of additional levels of control. To this end, we performed both in vitro and in vivo killing assays (Figure 1G and 1H). For the in vitro killing assay, we prepared engineered feedback loop CAR T cells which were either activated (PD1 KO PD1 KI + , circles) or left unstimulated (PD1 KO PD1 KI - , squares) to gauge their cytolytic potency (Figure 1G). As anticipated, stimulation boosted killing function of CAR T cells measured as faster clearance of CD19+ HEK target cells when compared to unstimulated control. Importantly, this result can be interpreted as a lowered activation state that will translate into reduced potency. Both observations are in line with the concept that antigen-mediated function can be regulated by cell activation state. Afterwards, we assessed cell killing efficacy in a xenograft Raji mouse model (Figure 1H). As presented, produced CAR T cells effectively cleared tumor cells within few weeks in a comparable manner regardless of CAR integration strategy. These data showcase the capability not only of PD-1 CAR KI but also the general approach of CRISPR-mediated gene engineering.

Based on the in vitro observation that the activation state may translate into CAR T cell potency on a population level, we expected to recapitulate similar effects in vivo. We hypothesized that re-challenge of mice few weeks after tumor clearance should lead to at least delayed response of activation-induced CAR T cells when compared to constantly CAR expressing counterparts (Figure 1I and 1J). To this end, we injected primary B cells derived from the same donor as CAR T cells to mimic a scenario where healthy B cells can be reinstated in patient's bloodstream after CAR-mediated tumor remission. Although we were not able to detect B cells directly at any time point due to limitation of our mouse model, we observed significant difference in tested CAR T cell populations over time. First, we observed fairly constant high frequencies of CAR+ cells that were generated by random integration even 5 weeks after initial injection and 3-4 weeks after tumor removal (Figure 1I). In contrast, engineered feedback loop CAR T cells content dramatically decreased after 2 weeks, which corresponded with tumor absence. Only in one outlier animal, both CAR T cell maintenance and tumor persistence were proportionally prolonged (Figure 1I). Interestingly, upon B cell re-challenge (d35) we observed a significant increase in conventional CAR T cells conversely to activation-inducible CAR T cells suggesting a more rapid antigen-specific response of cells with uncontrolled CAR expression (Figure 1J). The observed reaction is most likely due to the much higher content of former cells in the steady state. This observation is in line with our hypothesis that continuous unaltered presence of CAR T cells in the circulation will inhibit natural B cell restoration.

In conclusion, we demonstrated through in vitro and in vivo proof-of-concept experiments that CAR expression under activation-inducible endogenous promoter can result in regulated functional CAR T cells.

Discussion

In this manuscript we present an approach to control exogenous construct expression, and thus gained function of edited cells, via endogenous gene regulation in a form of an engineered feedback loop. In this setting, an artificial gene coding for construct-of-interest is introduced into the genome at the position that is managed by a promoter, the activity of which depends on cellular activation state. In the proof-of-concept experiments we focused on a clinically relevant scenario and utilized a CAR construct for T cell modification. We used several classical activation-dependent T cell markers to test our hypothesis such as, CD25, CD69 and PD-1. In this case, the CAR construct can be co-expressed (e.g.: CD69) or disrupt the targeted gene (e.g.: PD-1), thereby additionally improving anti-tumor potency depending on what is more desirable. Another engineering approach for physiologic expression of an artificial gene might be integration downstream of receptors that are downregulated during exhaustion, such as DNAM-1. This strategy could prevent terminal T cell exhaustion, thus increasing functional T cell persistence (Cella et al 2010). In the presented data, we targeted and disrupted PD-1 as well as CD69 genes.

In this activation-inducible system we were able to demonstrate that CAR content can be regulated on the population level by the cellular activation state. Moreover, this control follows the physiologic expression pattern of targeted proteins and upregulation dynamics upon indirect (non-CAR-mediated) stimulation. We demonstrated that this pattern can be maintained for at least three rounds of stimulation. The only detected divergence from physiological state was much higher frequency of CAR-positive T cells in unstimulated state. This higher-than-expected basal expression might be explained by tonic signaling through the CAR, albeit a non-clustering CAR was chosen. It has been described previously that ligand independent CAR oligomerization could drive T cells into exhaustion (Long et al 2015). Alternatively, elevated expression levels in steady state can be interpreted as artifacts of in vitro culture and culture-induced unspecific activation. Interestingly, in in vivo mouse models, CAR-positive T cell frequencies were resembling more closely the natural expression kinetics of targeted activation markers since CAR frequency declined in parallel to the tumor eradication and the anticipated physiological downregulation of PD-1.

The control over CAR content translated into different qualities of the function of T cell populations both in vitro and in vivo. In the in vitro killing assay, re-stimulated cells cleared antigen-expressing cells substantially faster than rested ones. Although we cannot formally exclude the possibility, we do not expect this to be an outcome of restimulation-induced phenotypic shift due to short time frame between conditions. Interestingly, in the Raji mouse model, we observed a pronounced correlation between T cell activity during tumor clearance and proportion of CAR-positive T cells detected in the blood. This correlation was even visible on the single-animal level where a mouse with longer persistence of malignant cells had high CAR T cell levels for prolonged period. However, in all mice that received the PD1 KO CAR KI cells, CAR frequency decreased substantially after tumor elimination in contrast to control animals receiving CAR T cells generated by lentiviral transduction. As a consequence, upon B cell injection, lentiviral control mice responded much faster as measured by a significant spike in CAR content. Nevertheless, we need to state that

our measurements focused on CAR T cell frequency in the blood, and we cannot exclude migration of these cells into other body niches.

These results imply a potential application of the engineered feedback loop as a temporal safety mechanism for CAR T cell therapies. If the construct knock-in is combined with simultaneous disruption of endogenous TCR, T cell activation will be maintained exclusively by CAR function and will cease upon depletion of all target cells in vivo. In other words, as long as CAR T cells find their target cells (e.g.: CD19 expressing tumor cells) they will remain active and perform their functions. After removal of all target cells, T cell activation and biologically associated CAR expression will decline. This will eventually render the CAR T cells unresponsive allowing healthy cell populations presenting same target antigen to recover (e.g.: healthy B cell compartment). Additionally, lack of endogenous TCR will prevent cell reactivation. Unresponsive CAR-TCR double-negative T cells should not affect patient's recovery and may die off due to their inability to receive tonic signaling.

We believe that the proposed editing logic can also be exploited in other clinically relevant scenarios in which on-target off-tumor killing makes temporal limited cell therapies desirable. The presented engineered feedback loop is a powerful tool combining state-of-the-art gene delivery methods to further refine T cell therapies. We hope our work will inspire further developments in the field of CAR T cells.

Methods

Material and Methods:

Primary cells

PBMCs were either isolated from fresh buffy coats via density gradient centrifugation using Biocoll (Biochrom) or selected from a Leukapheresis material using in house T cell selection technology ATC. Buffy coats were obtained from autologous adult female or male blood donors at the Institute for Anesthesiology, German heart center Munich (State of Bavaria and Technical University Munich). Written informed consent was obtained from each donor and usage of blood samples was approved according to national law by the local Institutional Review board and the declaration of Helsinki and Istanbul (Ethics committee of the faculty of Medicine, Technical University Munich: 360/13 and 55/14). Leukapheresis samples received from healthy donors were collected at the CCC Cellex Collection Center Dresden under the ethical quote (Ethical committee of the Technical University Dresden: EK309072016).

T cell culture and activation

Generally, T cells were enriched using in-house T cell selection technology (Radisch et al., 2022). Isolated T cells were cultured in serum free Medium (Thermo Fisher) in the presence or absence of cytokines. Enriched T cells were stimulated at specified time point with Expamers as previously described (Poltorak et al., 2020).

Generation of CRISPR/Cas reagents

crRNA sequences for gRNAs were 5-CGTCTGGGCGGTGCTACAACGTTT-TAGAGCTATGCT-3 for PDCD1 (targeting PDCD1) and 5-AGAGTCTCTCAGCTGGTACAGTTTTAGAGCTATGCT-3 for TRAC. RNPs were prepared according to manufacturers' recommendation. Briefly, 80 mM tracrRNA (IDT DNA) and 80 mM crRNA (IDT DNA) were incubated at 95°C for 5 min, then cooled to RT. 24 mM high fidelity Cas9 (IDT DNA) was added slowly to gRNA solution to yield RNPs with 12 mM Cas9 and 20 mM gRNA, as well as 20 mM electroporation enhancer (IDT DNA). RNPs were incubated for 15 min at RT and directly used for electroporation.

Double-stranded DNA PCR products were used for electroporation and HDR (Homology-directed repair). Plasmid DNA was amplified by PCR according to the following manufacturers' protocol. Thermal cycling was performed as following: 95°C and 30 s for initial denaturation followed by 34 cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 3 min and 72°C for 4 min as final extension step. Final PCR products were purified by Ampure XP beads according to manufacturer's protocol (Beckman Coulter). All HDR templates were titrated. Generally, electroporation of 1 µg DNA per 1×10^6 cells yielded best knock-in efficiencies.

Gene editing

Purified T cells were activated for 48 h with Expamers and 300 IU ml^{-1} recombinant human IL-2, 5 ng ml^{-1} recombinant human IL-7 (Peprotech) and 5 ng ml^{-1} IL-15. Expamer stimulus was disrupted by incubation with 1 mM D-biotin (Sigma). 1×10^6 cells were electroporated (pulse code EH100) with Cas9 ribonucleoprotein and DNA

templates in 20 ul Nucleofector Solution P3 (Lonza) with a 4D Nucleofector X unit (Lonza). After electroporation, cells were cultured in serum free media with 180 IU ml^{-1} IL-2 until first analysis on day 5 after editing. KI efficiency was measured using and anti-CAR antibody specific against the introduced scFv (Juno Therapeutics). KO was measured by staining surface expression of PD1 (BioLegend).

Genomic DNA Extraction and Knock-In validation

Genomic DNA was extracted (PureLink Genomic DNA Mini Kit, Invitrogen) from modified cells expression the transgenic and integrated receptor. PCRs were performed from the 5' homology arm to 3' homology arm to verify full construct integration. PCR amplifying the DNA sequence from outside the 5' genomic DNA into the integrated DNA Sequence validated the insertion into the desired loci. Purified PCR products were subsequently Sanger sequenced (Eurofins).

In vitro characterization

For in vitro expansion edited T cells were incubated with EBV-transformed B cells (LCL) in a 1:7 (E:T) ratio and the described protocol was performed accordingly (Turtle et al., 2016). CAR Frequency and absolute numbers were monitored and measured over indicated time points of expansion.

Sequential stimulation experiment was performed using expanded and rested engineered CAR T cells. Expamers stimulation was done as previously described. Stimulation signal was disrupted by adding 1 mM Biotin. Cells were subsequently washed and transferred into fresh media containing low dose IL-2 (25 IU ml^{-1}). Surface expression of activation markers was monitored by flow cytometry (see below) and cell count was measured (Nucleocounter, Chemotec).

For in vitro killing assay, xCELLigence RTCA System (ACEA Biosciences Inc.) was used. Specific lysis was measured targeting CD19 expressing Human Embryonic Kidney cells (HEK293-CD19$^+$). 2×10^4 Target cells were seeded into 96 well E-Plate (ACEA Biosciences Inc.) and rested overnight. Engineered CAR T cells were added in a 5:1 (E:T) ratio and incubated for indicated time. Changes in impedance signal were measured in real-time and analyzed using RTCA Software Pro (ACEA Biosciences Inc.).

Flow cytometry

Flow cytometry was performed as previously described (Poltorak et al., 2020; Radisch et al., 2022). Following antibodies were purchased from BioLegend and used for the analysis. CD3 (clone OKT3-PC7), CD4 (clone OKT4-BV421), CD8 (clone RPA-T8-BV510), CD45 (clone HI30), PD1 (clone EH12.2H7-FITC and -BV421), CD69 (clone FN50-APC/Fire), CD19 (clone HIB19-BV421 and -FITC), and CD25 (clone BC96-BV605). In addition, an anti-idiotype (CD19) antibody produced in-house coupled to APC (Bristol Myers Squibb) and Propidium Iodide (Thermo Fisher Scientific) was used.

In vivo tumor xenograft model

NSG-SGM3 (NOD.Cg-Prkdcscid Il2rgtmWjlTg (CMV-IL3, CSF2, KITLG) 1Eav/MloySzJ, NSGS, The Jackson Laboratory) mice were purchased from Jackson

Laboratory (The Jackson Laboratory) and bred in-house in a pathogen free facility at the Technical University Munich according to standard procedures. 6–10-week-old mice were used for experiments. All experiments were conducted as previously described and in the accordance with the guidelines of the Regierung von Oberbayern. All protocols were approved by Institutional Animal Care and Use Committee of the Regierung von Oberbayern (ROB-55.2-2532.Vet_02-17-138 and Vet_02-18-162).

B-cell rechallenge was executed by injecting 10×10^6 donor-matched B cells 2 weeks post tumor clearance.

Statistics

Flow cytometric data were analyzed using FlowJo software (FlowJo, LLC). Graphs and statistical analysis were generated using GraphPad Prism software (GraphPad Software).

Materials & Correspondence

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Data availability

The authors declare that all data generated or analyzed for this study are available within the paper and its supplementary information. Additional raw materials are available from the corresponding author upon reasonable request. Described findings are proprietary of Bristol-Myers Squibb Company and some information are considered a trade secret. Specific information about the technology can be made available upon reasonable request.

References:

- Kochenderfer et al 2010 Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19 DOI: 10.1182/blood-2010-04-281931
- Maude, S. L. et al. Tisagenlecleucel in Children and Young Adults with BCell Lymphoblastic Leukemia. N. Engl. J. Med. 378, 439–448 (2018).
- Killock, D. Anti-CD22 CAR T cells in ALL. Nat Rev Clin Oncol 17, 391 (2020)
- Munshi et al 2021 Idecabtagene Vicleucel in Relapsed and Refractory Multiple Myeloma
- Kalos M, et al. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leu- kemia.
- Paszkiewicz et al 2016 Targeted antibody-mediated depletion of murine CD19 CAR T cells permanently reverses B cell aplasia
- Rurik et al 2022 CAR T cells produced in vivo to treat cardiac injury
- Roybal et al 2016 Precision Tumor Recognition by T Cells with Combinatorial Antigen-Sensing Circuits
- Srivastava et al 2019 Logic-Gated ROR1 Chimeric Antigen Receptor Expression Rescues T Cell-Mediated Toxicity to Normal Tissues and Enables Selective Tumor Targeting
- Conley et al 2016 T Cells and Gene Regulation: The Switching On and Turning Up of Genes after T Cell Receptor Stimulation in CD8 T Cells
- Gros, A. et al. PD-1 identifies the patient-specific CD8+ tumor-reactive repertoire filtrating human tumors. J. Clin. Invest. 124, 2246–59 (2014)
- Han X, Wang Y, Wei J, Han W. Multi-antigen-targeted chimeric antigen receptor T cells for cancer therapy. J Hematol Oncol. 2019 Nov 29;12(1):128.
- Wang Z, Li N, Feng K, Chen M, Zhang Y, Liu Y, Yang Q, Nie J, Tang N, Zhang X, Cheng C, Shen L, He J, Ye X, Cao W, Wang H, Han W. Phase I study of CAR-T cells with PD-1 and TCR disruption in mesothelin-positive solid tumors. Cell Mol Immunol. 2021 Sep;18(9):2188-2198.
- Juillerat A, Tkach D, Yang M, Boyne A, Valton J, Poirot L, Duchateau P. Straightforward Generation of Ultrapure Off-the-Shelf Allogeneic CAR-T Cells. Front Bioeng Biotechnol. 2020 Jun 25;8:678.
  Adami A, Maher J. An overview of CAR T-cell clinical trial activity to 2021. Immunother Adv. 2021 Mar 9;1(1):ltab004.
- Fraietta, J.A., Nobles, C.L., Sammons, M.A. et al. Disruption of TET2 promotes the therapeutic efficacy of CD19-targeted T cells. Nature 558, 307–312 (2018).
- Ruella, M., Xu, J., Barrett, D. M., Fraietta, J. A., Reich, T. J., Ambrose, D. E., Klichinsky, M., Shestova, O., Patel, P. R., Kulikovskaya, I., Nazimuddin, F., Bhoj, V. G., Orlando, E. J., Fry, T. J., Bitter, H., Maude, S. L., Levine, B. L., Nobles, C. L., Bushman, F. D., Young, R. M., … Melenhorst, J. J. (2018). Induction of resistance to chimeric antigen receptor T cell therapy by transduction of a single leukemic B cell. Nature medicine, 24(10), 1499–1503.
- Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012 Aug 17;337(6096):816-21.

- Haberle V, Stark A. Eukaryotic core promoters and the functional basis of transcription initiation. Nat Rev Mol Cell Biol. 2018 Oct;19(10):621-637.
- Frigault MJ, Lee J, Basil MC, Carpenito C, Motohashi S, Scholler J, Kawalekar OU, Guedan S, McGettigan SE, Posey AD Jr, Ang S, Cooper LJ, Platt JM, Johnson FB, Paulos CM, Zhao Y, Kalos M, Milone MC, June CH. Identification of chimeric antigen receptors that mediate constitutive or inducible proliferation of T cells. Cancer Immunol Res. 2015 Apr;3(4):356-67.
- Eyquem J, Mansilla-Soto J, Giavridis T, van der Stegen SJ, Hamieh M, Cunanan KM, Odak A, Gönen M, Sadelain M. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature. 2017 Mar 2;543(7643):113-117.
- Kloss CC, Condomines M, Cartellieri M, Bachmann M, Sadelain M. Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells. Nat Biotechnol. 2013 Jan;31(1):71-5.
- Poltorak MP, Graef P, Tschulik C, Wagner M, Cletiu V, Dreher S, Borjan B, Fraessle SP, Effenberger M, Turk M, Busch DH, Plitzko J, Kugler DG, Ragan S, Schmidt T, Stemberger C, Germeroth L. Expamers: a new technology to control T cell activation. Sci Rep. 2020 Oct 20;10(1):17832.
- Schober K, Müller TR, Gökmen F, Grassmann S, Effenberger M, Poltorak M, Stemberger C, Schumann K, Roth TL, Marson A, Busch DH. Orthotopic replacement of T-cell receptor α- and β-chains with preservation of near-physiological T-cell function. Nat Biomed Eng. 2019 Dec;3(12):974-984.
- Radisch S, Poltorak MP, Wagner M, Cletiu V, Radisch C, Treise I, Pann S, Weigt A, Artner S, Dreher S, Fechner F, Borjan B, Fraessle SP, Effenberger M, Benke E, Navratil G, Hentschel N, Busch DH, Schmidt T, Stemberger C, Germeroth L. Next Generation Automated Traceless Cell Chromatography Platform for GMP-Compliant Cell Isolation and Activation. Sci Rep 2022.
- Salter AI, Ivey RG, Kennedy JJ, Voillet V, Rajan A, Alderman EJ, Voytovich UJ, Lin C, Sommermeyer D, Liu L, Whiteaker JR, Gottardo R, Paulovich AG, Riddell SR. Phosphoproteomic analysis of chimeric antigen receptor signaling reveals kinetic and quantitative differences that affect cell function. Sci Signal. 2018 Aug 21;11(544):eaat6753.
- Turtle CJ, Hanafi LA, Berger C, Gooley TA, Cherian S, Hudecek M, Sommermeyer D, Melville K, Pender B, Budiarto TM, Robinson E, Steevens NN, Chaney C, Soma L, Chen X, Yeung C, Wood B, Li D, Cao J, Heimfeld S, Jensen MC, Riddell SR, Maloney DG. CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. J Clin Invest. 2016 Jun 1;126(6):2123-38.
- Bishop DC, Clancy LE, Simms R, Burgess J, Mathew G, Moezzi L, Street JA, Sutrave G, Atkins E, McGuire HM, Gloss BS, Lee K, Jiang W, Maddock K, McCaughan G, Avdic S, Antonenas V, O'Brien TA, Shaw PJ, Irving DO, Gottlieb DJ, Blyth E, Micklethwaite KP. Development of CAR T-cell lymphoma in 2 of 10 patients effectively treated with piggyBac-modified CD19 CAR T cells. Blood. 2021 Oct 21;138(16):1504-1509.
- Roth TL, Puig-Saus C, Yu R, Shifrut E, Carnevale J, Li PJ, Hiatt J, Saco J, Krystofinski P, Li H, Tobin V, Nguyen DN, Lee MR, Putnam AL, Ferris AL, Chen JW, Schickel JN, Pellerin L, Carmody D, Alkorta-Aranburu G, Del

Gaudio D, Matsumoto H, Morell M, Mao Y, Cho M, Quadros RM, Gurumurthy CB, Smith B, Haugwitz M, Hughes SH, Weissman JS, Schumann K, Esensten JH, May AP, Ashworth A, Kupfer GM, Greeley SAW, Bacchetta R, Meffre E, Roncarolo MG, Romberg N, Herold KC, Ribas A, Leonetti MD, Marson A. Reprogramming human T cell function and specificity with non-viral genome targeting. Nature. 2018 Jul;559(7714):405-409.

Figure 1. CAR expression can be linked to T cell activation. (A) Schematic representation of CAR expression under control of activation-dependent promoter. **(B)** Physiological expression kinetics of PD1, CD25 and CD69 activation markers. Graph presents marker expression over time from 3 donors. Cells were restimulated on d6 (arrow) after initial stimulation on d0. Errors bars represent mean±SD. **(C)** CAR can be successfully knocked-in into PD1 locus. Set of representative dot plots and histogram are shown. Plots pre-gated on living, single CD45+ cells. **(D)** CAR expression under PD1 locus is activation-dependent upon polyclonal anti-CD3/CD28 stimulation. Graphs display CAR expression as arbitrary units (A.U.) in rested or restimulated samples. CAR constructs targeted into TRAC locus as well as expressed via lentiviral (LV) random integration were used as a control. Bars represent mean±SD. The significance was analyzed using unpaired Student's t test. **(E)** CAR T cells can be expanded via antigen specific stimulation. Graph depicts fold expansion upon two rounds of antigen-specific stimulation. **(F)** Antigen-specific expansion leads to increased CAR T cell content in all samples, whereas polyclonal restimulation enhances CAR T cell content in activation-induced CAR expression setting only. Graphs display indicated marker expression as frequencies in rested or restimulated samples. CAR constructs targeted into PD1 locus as well as expressed via lentiviral (LV) random integration are depicted. Arrows indicate start (d0) and end (d2) of stimulation period. Error bars represent mean±SD. The significance was analyzed using paired Student's t test. **(G)** Activated engineered feedback loop CAR T cells show faster in vitro killing compared to rested counterpart. Graph shows impedance measurement as a read out of antigen specific killing of target cells. Untransduced T cells (neg control), target cells only (HEK control), PD1 KO only T cells (PD1 KO), CAR T cells LV-transduced (LV control) as well as rested (PD1 KO PD1 KI -) and polyclonally restimulated (PD1 KO PD1 KI +) engineered feedback loop CAR T cells were used. **(H)** Engineered feedback loop CAR T cells are as functional as state-of-the-art CAR T cell product in an in vivo mouse model. Tumor was detected using IVIS bioluminescence measurements. Luminescence images from one representative experiment are shown. **(I)** Engineered feedback loop CAR T cells can help restore B cell compartment after complete tumor eradication. CAR T cells content in blood of each individual mouse was calculated using cell frequencies measured via flow-cytometry. Cells were pre-gated on living lymphocytes. Box highlights cell frequencies after B cell re-challenge (d35). Mouse with prolonged tumor burden highlighted by star in H and I. **(J)** B cell re-challenge on day 35 leads to rapid increase in LV-transduced CAR T cell content while engineered feedback loop CAR T cell content remains unchanged. Delta calculated based of CAR T cell frequencies between day 28 (tumor clearance) and day 45 (post re-challenge). Error bars represent mean±SD. The significance was analyzed using unpaired Student's t test.