# Allogeneic TCRα/CS1 double knockout T-cells bearing an anti-CS1 chimeric antigen receptor: An improved immunotherapy approach for the treatment of Multiple Myeloma

Poster #2289

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## #1 Introduction

- Multiple Myeloma (MM) is a B-cell neoplasia characterized by clonal expansion of malignant plasma cells in the bone marrow.

- Although currently available therapies can improve patient's overall survival, MM still remains incurable in most of the cases.

- The use of autologous chimeric antigen receptor (CAR)-redirected T-cells has allowed to achieve long-term durable remissions in patients with B cell leukemia, indicating that CAR technology may become a new alternative in cancer treatment.

**- In the present work we have assessed the feasibility of CAR-mediated targeting of the CS1 antigen (SLAMF7), which is highly expressed on tumor cells from most patients with MM.**

**- Expression of CS1 on normal CD8+ T-cells is potentially an obstacle for the development of CAR T-cells against this protein, since antigen-expressing T cells will be targeted, impacting both on the number and the phenotype of the final CAR T-cell population.**

- Transcription Activator-Like Effector Nuclease (TALEN) gene editing technology was used to inactivate the CS1 gene in T-cells, prior to transduction with a lentiviral vector encoding an anti-CS1 CAR. Anti-tumor activity of gene-edited CAR T-cells was validated in vitro.

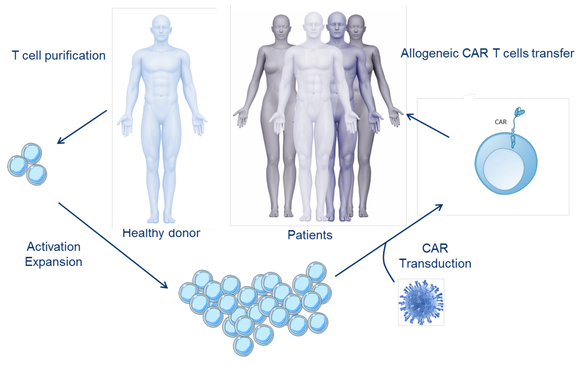
- Gene editing technology was also used here to inactivate the TCRα constant (TRAC) gene, to minimize the potential for engineered T-cells to mediate Graft versus Host Disease (GvHD).

- Finally, we evaluated the in vivo activity of double knockout CAR T-cells (UCARTCS1) by performing experiments in an orthotopic MM mouse model, showing that CS1/TCRα disrupted T-cells were able to mediate an *in vivo* anti-tumoral activity.

**Our results show that multiplex genome editing is possible and can lead to the production of double KO TRAC/CS1 T-cells, allowing large scale manufacturing of allogeneic, non alloreactive CS1 specific T-cells with enhanced anti-tumor activity.**

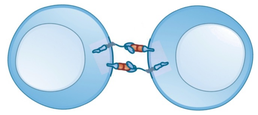
## #2 Allogeneic approach to target CS1

**One of the key barriers for adoptive transfer of 3rd party CAR T-cells can be overcome via TALEN® gene editing technology to disrupt expression of the TCR, allowing the use of any donors' T-cells and minimizing the risk of GvHD.**
**This technology can also be used to inactivate the CS1 gene in the same cell, allowing a more efficient production of allogeneic anti-CS1 CAR T-cells**

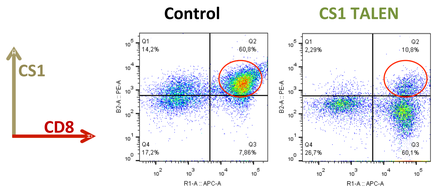



## #3 CS1 gene inactivation in human T-cells

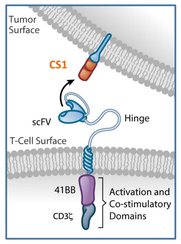
CS1 is expressed on CD8+ T-cells, and these cells can be targeted during amplification of CAR T-cells



TALEN® targeting the coding sequence of the human CS1 gene were designed, and the corresponding mRNAs were transfected in primary T-cells using PulseAgile electroporation technology.
**Flow cytometry analysis revealed high levels of CS1 gene inactivation in CD8+ T-cells.**



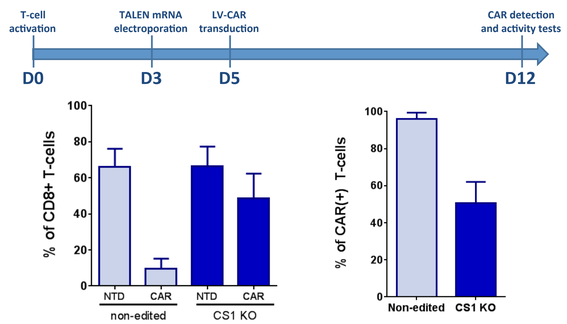

## #4 Generation of anti-CS1 CAR




An scFv targeting the extracellular domain of the human CS1 antigen (SLAMF7) was used to construct a chimeric antigen receptor containing a 41BB co-stimulatory domain and the CD3ζ activation domain.

## #5 CS1 CAR expression in human T-cells

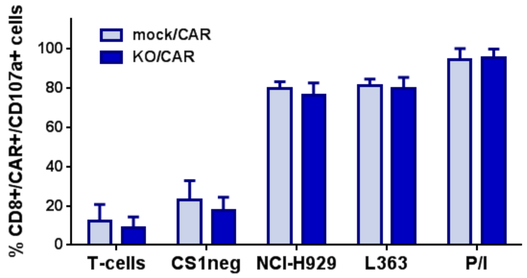
T-cells were transfected with mRNAs encoding the TALEN® targeting the CS1 gene (or mock transfected), and transduced 2 days later with the lentiviral vector encoding the anti-CS1 CAR.
7 days after transduction T-cells were analyzed for CD8 and CAR expression by flow cytometry.



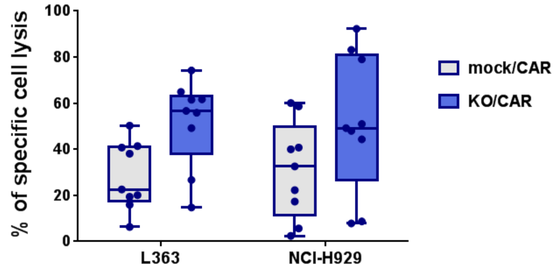

**The percentage of CD8+ cells is dramatically reduced in non-edited cells upon anti-CS1 CAR expression, indicating that CD8+ cells are being targeted during the expansion step. Furthermore, a concomitant enrichment of CAR+ cells is observed in these cells compared to CS1 gene-edited cells.**

## #6 anti-CS1 CAR activity is enhanced in CS1 KO T-cells

Degranulation activity upon co-culture of CAR T-cells with CS1 expressing cells (NCI-H929 or L363) was measured by detecting CD107a expression in CD8+/CAR+ cells after 6h of co-culture. $CS1_{neg}$ cells were used as a control.
**CS1 KO cells are able to degranulate in the presence of the CS1 antigen at the same levels that non-edited cells expressing the same CAR.**

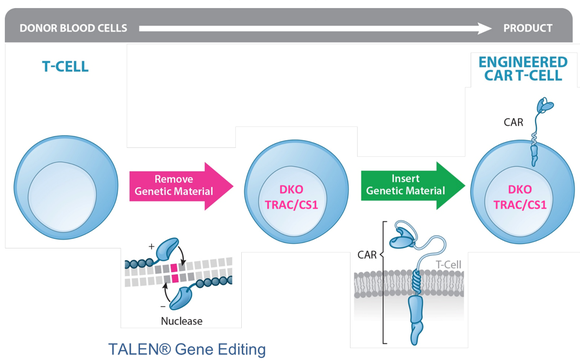



Cytotoxic activity against L363 and NCI-H929 cells was also measured by flow cytometry, upon co-culturing the effector and target cells for 4h.
Activity is normalized to consider an equal transduction efficiency between mock and CS1 KO T-cells.
**Even if differential activities are observed among the 9 donors tested, CS1 KO cells show a higher cytotoxic activity when compared to mock transfected cells.**



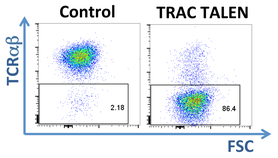

## #7 Generation of UCARTCS1 cells

UCARTCS1 cells can be produced using cells from a healthy donor in a process designed for GMP compatibility.
The CS1 KO is performed prior to CAR transduction, and the TCRαβ KO cells are purified at the end of the production process.




TALEN® Gene Editing

## #8 TCRα/CS1 gene inactivation in human T-cells

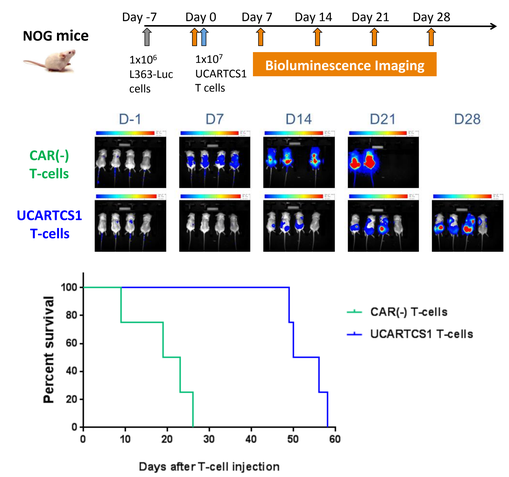



TALEN® targeting the CS1 gene were co-transfected with those targeting the human TCRα gene. The TCRαβ(-) cells were purified and the % of NHEJ at each locus was quantified by DeepSeq.

| LOCUS | % NHEJ | INS | DEL | WT | UA |
|---|---|---|---|---|---|
| CS1 | **85.9 ± 5.2** | 2.8 ± 0.7 | 83.2 ± 5.1 | 13.9 ± 5.2 | 0.8 ± 0.2 |
| TRAC | **89.4 ± 3.8** | 3.6 ± 0.1 | 76.0 ± 1.6 | 9.3 ± 3.5 | 12.9 ± 1.9 |

## #9 UCARTCS1 anti-tumor activity in vivo

NOG mice were sublethally irradiated (1,44 Gy) 8 days before injection of T-cells. At Day (-7) $10^6$ L363-Luciferase cells/mice were iv injected.
Mice were then infused with CAR(-) T-cells or UCARTCS1 cells (CS1/TCRα KO, CAR+ T-cells). Bioluminiscent signal was assessed at D(-1), D7, D14, D21 and D28 post injection of T-cells.




**UCARTCS1 cells display anti-tumor activity in vivo and increase mice overall survival**

## #10 Conclusions and Perspectives

**1) We show here that TALEN®–mediated disruption of the CS1 gene in T-cells is efficient and improves in vitro activity of T-cells harboring an anti-CS1 CAR.**

**2) We have previously demonstrated that TALEN® mediated inactivation of the TCRα constant (TRAC) gene can be achieved at high frequencies and eliminate the potential for edited T-cells to mediate Graft versus Host Disease (GvHD).**

**3) Multiplex genome editing can lead to the production of double KO (TRAC and CS1) T-cells, allowing large scale manufacturing of allogeneic, non alloreactive CS1 specific T-cells that could display enhanced anti-tumor activity.**

**4) Gene editing technology offers the possibility of developing an off-the-shelf CAR T-cell based frozen product that would be immediately available for administration to a large number of MM patients.**

# Abstract number 2307

# Improved CAR safety by a non-lethal switch to control CAR activity at the T-cell surface membrane

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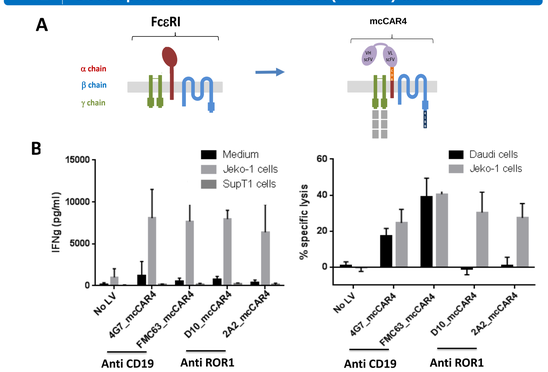
## #1 Abstract

Adoptive immunotherapy using engineered T-cells has emerged as a powerful approach to treat cancer. The potential of this approach relies on the ability to redirect the specificity of T cells through genetic engineering. Novel specificities in T cells have been typically implemented through the genetic transfer of the so-called chimeric antigen receptors (CARs). CARs are synthetic receptors that associated an extracellular targeting moiety with one or more intracytoplasmic signaling domain derived from lymphocyte activation receptors. Present CAR architectures are designed to combine all relevant domains within a single polypeptide, thereby; they combines advantages of MHC unrestricted target recognition to the potent native effector mechanisms of the T cell. Although adoptive transfer of CAR T cells is proven to be an effective strategy to cancer therapy, potential adverse effects such as Cytokines Release Syndrome (CRS) and/or the risk of on-target off-tumor targeting are still a major concern. To date only Suicide mechanisms that can eradicate the engineered T-Cell “at will” or mRNA CAR transfection approaches have been proposed to addresses this safety issue.

Here, we describe the development of a small molecule based switch-on technology to control the surface presentation of a chimeric antigen receptor in T-cells. By grafting protein domains that can interact upon addition of a small molecule drug in the hinge domain of the CAR architecture, we are able to turn a T-cell endowed with an engineered CAR from an off-state to an on-state, in term surface presentation as well as for its specific cytolytic properties. This system offers the advantage of a ”transient CAR T-cell” for safety while letting open the possibility of multiple cytotoxicity cycles using a small molecule drug. This non-lethal control system of CAR engineered T-cells represents an important advance for the safety of this technology.

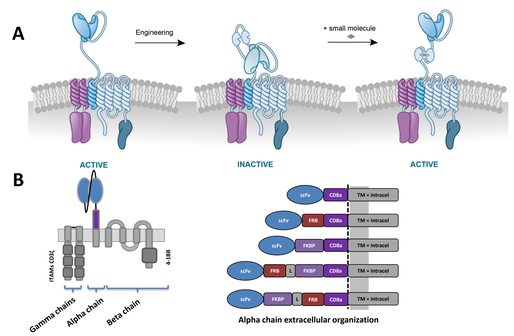
## #2 Development of a multichain CAR (mcCAR) derived from FceRI




**Functional design of multichain CAR for adoptive immunotherapy.**

**A:** To generate a multi-chain CAR (mcCAR) construct based on FceRI receptor, the extracellular domain of the alpha chain was deleted and replaced by the CD8a stalk and an scFv specific for a given antigen (CD19) to constitute an antigen-recognition domain . Several variants were also designed (type, number and position of the added sequences) which showed show significant and specific degranulation, cytokine secretion and cytotoxicity against Daudi cells (data not shown). We focused on mcCAR4 containing CD3ξ ITAMs and 4-1BB intracellular domain.
**B:** T cells bearing stably expressed mcCAR4 show potent activity against target cells in vitro.

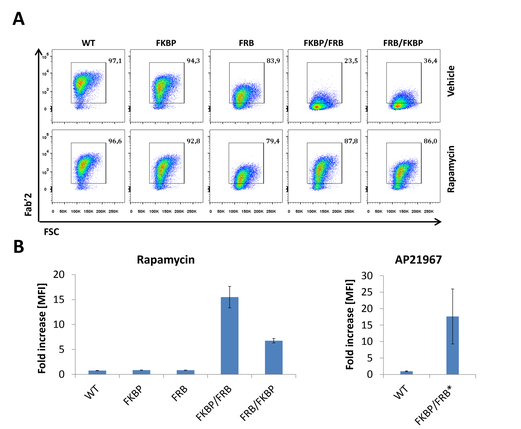
## #3 Principle and design of mcCAR with inducible activity




**Schematic representation of the engineered mcCAR.**

**A:** Principe of inducible conformational changes in the hinge domain. The CAR T-cell performance is intimately linked to an optimal interaction of the scFv to the targeted antigen. We thus conceived a system where controlled variations in the conformation of the hinge that separates the scFv from the cell membrane could be obtained upon addition of a small molecule.
**B:** Design of the hinge domains that incorporate FRB and/or FKBP domains. To switch the scFv/antigen interaction between on/off states, we inserted either the FRB, the FKBP12, or fusion of the FRB and FKBP12 between the CD8a hinge and the scFv domains. All constructs are based on mcCAR4

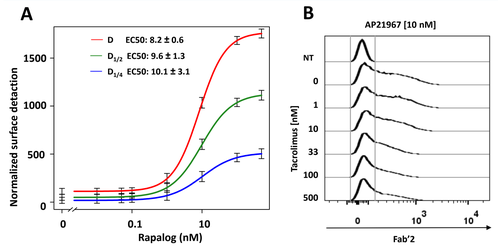
## #4 Surface detection of FKPB/FRB-CAR




**Surface detection of the engineered CAR in response to small molecules.**

Primary T cell with were transfected with mRNAs encoding each chain of the multichain CAR (mcCAR). Upon addition of rapamycin or rapalog, we monitored conformational changes of the extracellular hinge domain by tracking the Fab’2 domain of the CD19-targeting scFv (100 nM, 20 h) (**A**). While the addition of rapamycin had no effects on the mcCAR, FRB-mcCAR and FKB-mcCAR), it strongly improved (up to 15 fold, **B**) the surface detection of the FKBP/FRB-mcCAR and FRB/FKBP-mcCAR constructs turning the system from an off to an on state.

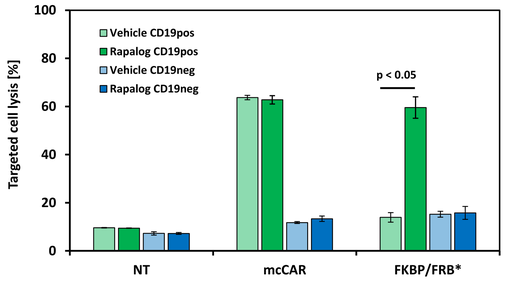
## #5 Surface expression is dose dependant (rapalog AP21967)




**Characterization of the small molecule switch-on system.**

**A:** To evaluate the AP21967 usable dose range for the switch-on system we performed a dose response assay . T-cells were transfected with three doses (D, $D_{1/2}$ and $D_{1/4}$) of mRNA coding for the engineered CAR and were treated with increasing amount of AP21967 rapalog. The Fab’2 region of the scFv is detected. The results we obtained indicated a maximum signal induction at 100 nM and an EC50 value of approximately 10nM (8.2-10.1 nM) that was independent from the amount of transfected engineered CAR. Remarkably, the EC50s are in range with rapamycin concentrations reported in peripheral blood or tumor tissue of patients, suggesting that the switch-on system may be sensitive to clinically relevant concentration.
**B:** Competition experiment between AP21967 (10 nM) and tacrolimus (0 to 500 nM). N=2, error bars denote s.d. The possibility to further modulate the system using alternative small molecule competitors offers additional control of the engineered CAR T-cells. To illustrate the possibility to tune the amount of CAR locked in an on-state at the cell surface, we used the tacrolimus (FK506), a small molecule known to bind to the FKBP12 without enabling to form a complex with the FRB. AP21967 (or rapamycin) and tacrolimus have identical FKBP12 binding core and compete for the same binding site within the FKBP moiety. T-cells transfected with the engineered CAR were incubated with a fixed amount of AP21967 (10 nM) and increasing amount of tacrolimus (0 to 500 nM) and the surface labelling of the scFv was recorded. Addition of increasing amounts of tacrolimus competed with AP21967 for the binding site on FKBP and decreased the surface detection of the CAR

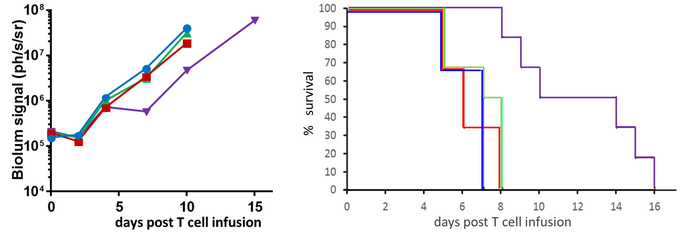
## #6 FKPB/FRB-CAR T-cell cytotoxic activity




**Specific cytolytic properties of the engineered CAR T-cells.**

The effect of the AP21967 rapalog on the cytolytic capacites of the of the CAR T cells toward model antigen presenting cell was assessed in a flow-based cytotoxicity assay. The $CD19^{pos}$ and a $CD19^{neg}$ target cell viability was measured after coculture with engineered CAR T-cells in presence or absence of AP21967. Effector/target ratios was set to 20:1. NT represents non-transfected T-cells, N=2, error bars denote s.d.
The engineered FKBP/FRB-CAR T-cell presented a significant cell lysis activity only in presence of the AP21967. We showed that the hinge engineering did not impair the specificity feature of the engineered T-cell as no cytotoxicity was observed on $CD19^{neg}$ target cells

## #7 FKPB/FRB-CAR T-cell *in vivo* activity against CD123+ tumors




**G1:** T cell-Vehicle **G2:** T cell + Rapalog **G3:** Switch On-Vehicle **G4:** Switch On + Rapalog

**In vivo inducible activity cytolytic properties of the engineered CAR T-cells.**

The effect of the AP21967 rapalog on the cytolytic capacites of the of the CAR T cells towards model antigen presenting cell was assessed *in vivo* using human tumor cell line xenograft into immunodeficient NOG mice. Firefly luciferase-expressing MOLM13 cells (0.25 x $10^6$ cells/mouse) were injected intraveinously. 7 days later, mice received a single i.v. infusion of 15 x $10^6$ T cells containing 10 x $10^6$ FKLBP/FRB-CAR+ cells or non-transduced control T cells. Following T cell injection, AP21967 treatment started and consisted of2 daily IP injections at 3 mg/kg/injection for 10 consecutive days. Vehicle was used as control for AP21967. Bioluminescence was analyzed 2 to 3 times a week until sacrifice of the animals.

## #7 Conclusions

Recent clinical implementation of adoptive cell transfer of CAR engineered T-cells has proven a powerful and successful approach to cancer immunotherapy. The capability to control T cells endowed permanently with such molecules is a key feature concerning the safety of this technology.

Here, we describe a strategy to create a small molecule based switch technology to control the engineered CAR T-cells. Our approach is based on inducing deliberate conformational changes in the hinge domain that separate the scFv from the cell membrane. Although our approach is applicable to current CAR design (data not shown), we chose to implement this strategy in a novel CAR architecture that relies on the FceRI receptor scaffold. The particularity of this design resides in the possibility to split or combine different key functions of a CAR such as activation and costimulation within different chains of a receptor complex, mimicking the complexity of the TCR native architecture.

We first showed that the multichain CAR architecture could redirect T-cells to various antigens. Second, we showed that the hinge engineering approaches allowed to turn a T-cell endowed with an engineered CAR from an off-state to an on-state. By controlling the scFv presentation at the cell surface upon addition of the small molecule, our all-in-one system allowed to further induce the cytolytic properties of the engineered T-cell. Finally, we provide initial in vivo data showing anti-tumor activity of our inducible CAR. This activity is not maintained once the small molecule treatment is interrupted.

Overall, this non-lethal system offers the advantage of a ”transient CAR T-cell” for safety while letting open the possibility of multiple specific cytotoxicity cycles using a small molecule drug. Finally, similar strategies that perturb optimal presentation of the antigen targeting moiety of CAR may be easily implemented in order to promote a small molecule off-switch.