Integration of Dual Signal Input Strategies in Novel Chimeric Antigen Receptors to Control the CAR T-Cell Functions

Alexandre Juillerat, Alan Marechal, Jean Marie Filhol, Yannick Valogne, Valérie Guyot, Anne-Sophie Gautron, Julien Valton, Aymeric Duclert, Laurent Poirot and Philippe Duchateau

Cellectis, Inc., 430E, 29th Street, NYC, NY 10016, USA - Cellectis S.A., 8 rue de la Croix Jarry, 75013 Paris, France

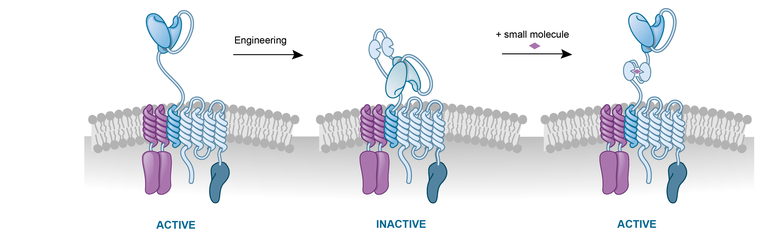
#1 | Abstract

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 composed of an extracellular targeting moiety and 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 combine 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 cancer therapy, potential adverse effects such as cytokine release syndrome (CRS) and/or the risk of on-target off-tumor targeting are still a major concern.

Synthetic biology applies many of the principles of engineering to the field of biology in order to create biological devices which can ultimately be integrated into increasingly complex systems. Our ability to engineer synthetic systems in primary T-cells that function as Boolean logic gates responding to multiple inputs would benefit adoptive immunotherapy using engineered T-cells. Exogenous or endogenous environmental signal integration by a modular AND gate may represent an important advancement in improving our control of the safety of the CAR T-cell technology.

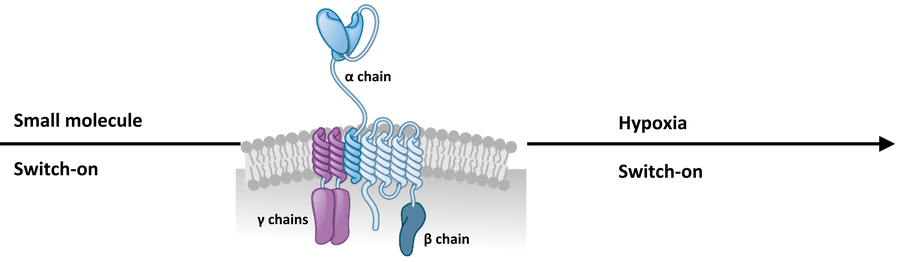
Here, we describe the development of novel CAR designs that integrate new components directly within the CAR architecture to improve our capacity to spatiotemporally control and switch the CAR T-cells functions between on and off states. In particular, we showed that such a system can be engineered to control the CAR through addition of an exogenous small molecule (Rapamycin or synthetic rapalogs) ultimately inducing the cytolytic properties of the engineered T-cell. We showed that oxygen levels can be used to trigger the CAR surface presentation, creating a so called “self-decision making” CAR T-cell.

#3| Exogenous small molecule CAR T-cell control



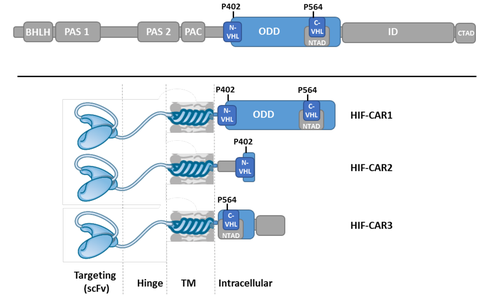
Principe of the switch-on strategy integrated in the hinge domain. The CAR T-cell performance is intimately linked to an optimal interaction of the scFv to the targeted antigen. We thus conceived a system where controlled variations in hinge that separates the scFv from the cell membrane could be obtained upon addition of a small molecule. 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.

#2| The multichain CAR scaffold



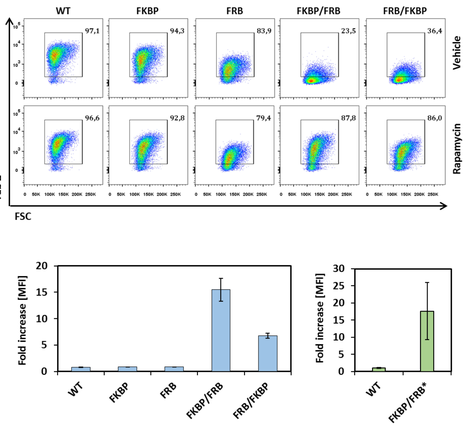
To generate a multi-chain CAR (mcCAR) construct based on FceRI receptor, the extracellular domain of the alpha chain was deleted and replaced by the CD8a stalk and an scFv specific for a given antigen to constitute an antigen-recognition domain. The native activation domains on the gamma and beta subunits were substituted by the intracytoplasmic signaling region of the ζ-chain of the CD3−T cell receptor and by the signaling domains from co-stimulatory 4-1BB (CD137) respectively. **We aimed at engineering a T-cell that requires a double input, constituted of the antigen recognition and either hypoxic microenvironment or a small molecule, to obtain an effective output, the T-cell activation and subsequent cytolytic properties.**

#4 | Endogenous microenvironment CAR T-cell control



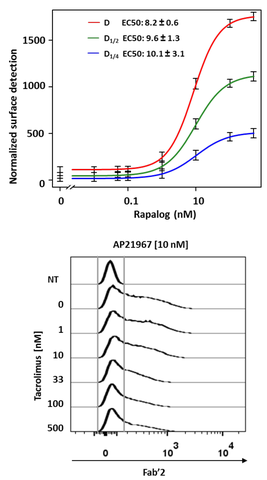
We used oxygen sensitive subdomains of the human HIF1α, excluding the transcription activation domains, to create a CAR that will be responsive (protein degradation) to oxygen variation. In particular, three fragments of the HIF1 α that contains key Proline residues (P402 and P564) known to be hydroxylated in normoxia and involved in interactions with the von Hippel-Lindau tumor suppressor E3 ubiquitin ligase (VHLE3) multi-protein complex were fused to the mcCAR α chain.

#3a| Controlled CAR surface presentation



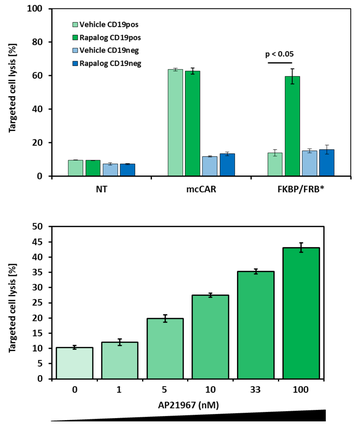
Engineered CAR T cells are responsive to addition of a small molecule. (A) Primary T cell with were transfected with mRNAs encoding each chain of the multichain CAR (mcCAR). Upon addition of rapamycin or rapalog, we monitored the surface presentation of the extracellular hinge domain by tracking the Fab’2 domain of the CD19-targeting scFv (100 nM, 20 h). 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. (C) Using a mutant o FRB allows

#3b| Switch-on/off properties



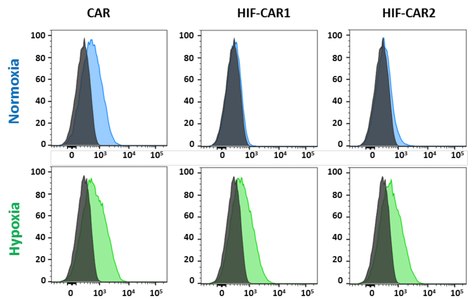
Rapalog EC50 and modulation of the response with an alternative small molecule (tacrolimus). (A) Remarkably, the EC50s (8.2-10.1 nM) are in range with rapamycin concentrations reported in peripheral blood or tumor tissue of patients. **(B)** To illustrate the possibility to tune the amount of CAR locked 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. Addition of increasing amounts of tacrolimus competed with AP21967 for the binding site on FKBP and decreased the surface detection of the CAR .

#3c| Controlled CAR T-cells cytotoxicity



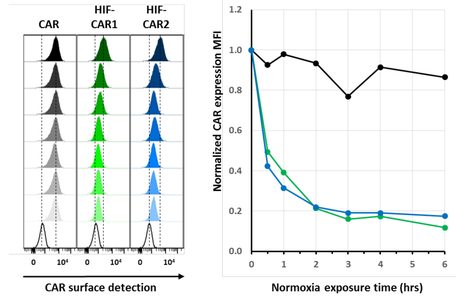
Engineered CAR T cells display antitumor cytotoxicity upon addition of AP21967. (A) The engineered FKBP/FRB-CAR T-cell presented a significant cell lysis activity only in presence of the AP21967. We showed that the hinge engineering did not impair the specificity feature of the engineered T-cell as no cytotoxicity was observed on CD19^{neg} target cells. **(B)** We found that the level of target cell killing correlated, as expected, with variation of the AP21967. We further calculated an EC50 of approximately 10nM (12.7 nM), in range of the one determined using the surface detection.

#3a| Controlled CAR surface presentation



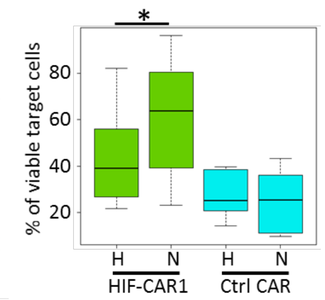
Low oxygen concentration as an input: CAR surface expression. Activated primary T-cells transfected with the different CARs were incubated for 20 hours either in classical cell culture conditions or in an artificially created hypoxic environment. We then monitored the surface presentation of the CAR and more specifically of the alpha chain containing the oxygen sensitive domain, by tracking the Fab’2 domain of the targeting scFv. As expected, the variation in oxygen concentration only weakly influenced the surface detection of the scFv for the classical control CAR. However, the hypoxic condition markedly improved the surface presentation of HIF-CARs containing the large (HIF-CAR1) or Nter (HIF-CAR2) portion of HIF1α.

#3b| Switch-off properties



HIF-CAR are rapidly switching-off in after removal of inducing signal. One key feature of such reactive switch systems is their ability to quickly return to their off state in absence of the inducing signal (hypoxia). In particular, this characteristic would be of prime interest to protect distant healthy tissues form off-tumor/on-target effects. We determined that the CAR surface expression was decreased by 80% in approximately 2 hours for the HIF-CAR1 and HIF-CAR2, considering the mean fluorescence intensity.

#3c| controlled cytotoxicity



Combinatorial input signal is required for enhanced cytolytic properties. We found that the capacities of the engineered HIF-CAR T-cells to kill target cells were significantly improved (p-values = 0.0276, E/T ratio = 10) in hypoxia versus normoxia. In contrast, as expected, we did not observed a significant improvement in the viability of the target cells in the hypoxic condition relative to the normoxic conditions for the classical CAR.

#4| 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 the integration of dual signal input strategies in a novel chimeric antigen receptors to extend the possibilities to control the CAR T-Cell functions. We reported a small molecule based switch technology to control the engineered CAR T-cells. 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. We also provide the proof of principle of engineering a CAR scaffold to create an integrated oxygen-based self decision making T-cell, that allows tuning the cytolytic properties of CAR T-cells depending on the microenvironment. This work also provides a basic framework to use a multi chain CAR as a platform to create a next generation of smarter CAR T-cells.

Engineered CAR T Cell Platform for Allogeneic Combination Immunotherapy

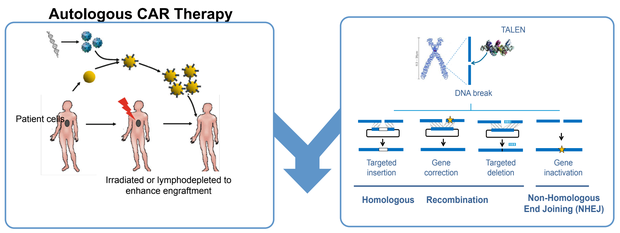
Laurent Poirot, Alexandre Juillerat, Julien Valton, Anne-Sophie Gautron and Philippe Duchateau

CELLECTIS, 8 rue de la Croix Jarry, 75013 Paris, France. Email: philippe.duchateau@cellectis.com

#1 Abstract

Chimeric antigen receptor (CAR)-redirected T-cells have given rise to long-term durable remissions and remarkable objective response rates in patients with refractory leukemia, raising hopes that a wider application of CAR technology may lead to a new paradigm in cancer treatment. Similarly, transcription activator-like effector nuclease (TALEN™)-mediated gene editing has emerged as powerful strategy to introduce targeted mutations and holds great promise in therapeutics and offers multiple opportunities to improve CAR T-cell therapies. The knockout of the TCR alpha gene eliminates TCR expression and abrogates the donor T-cell's potential for graft-versus-host disease (GvHD) while maintaining a potent anti-tumoral activity. Thus it is possible to manufacture T-cells from third-party healthy donors to generate allogeneic "off-the-shelf" engineered CAR T-cells. Disruption of CD52 or deoxycytidine kinase genes may be a useful approach to makes T-cells compatible with concurrent oncology treatments such as alemtuzumab or Fludarabine. Here, we present *in vitro* and *in vivo* proof of concepts demonstrating the potential of TALEN™-mediated gene editing for adoptive T-cell therapy.

#2 Sharpening the power of T-cell by editing the genome

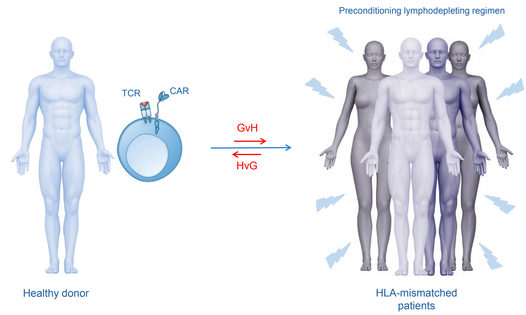


Allogeneic, non alloreactive	Off-the-shelf product (KO TCR)
Drug resistance	Combination therapies (KO CD52, PSMB5*) Enhanced engraftment (KO DCK)
Resistant to tumor inhibition	Enhanced efficacy (KO PD1, KO CTLA4,…)
Suppressed cross T-cell reaction	Better suited for specific tumors (KO CD38, KO CS-1)

TALEN enable us to efficiently modify or inactivate any target gene in a highly efficient, specific and precise manner. Thus, We can engineered product candidates to achieve desired clinical attributes. By editing and engineering the genomes of the T-cell, we have been able to establish "smart" CAR-T cells that include the above traits.

#3 Allogeneic CAR T-Cell Therapy

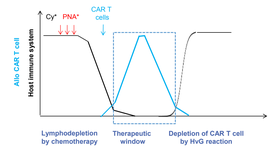
A NEED TO CONTROL HOST VERSUS GRAFT (HVG) AND GRAFT VERSUS HOST (GVH) REACTIONS



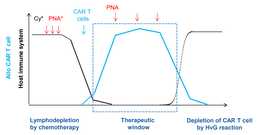
#4 Controlling the therapeutic window

CONTROLLING THE THERAPEUTIC WINDOW BY TUNNING LYMPHODEPLETION

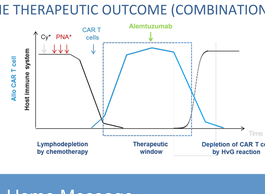
- TO IMPROVE CAR T CELLS PROLIFERATION (PRECONDITIONING)



- TO CONTROL THE EXTENT OF THE THERAPEUTIC WINDOW

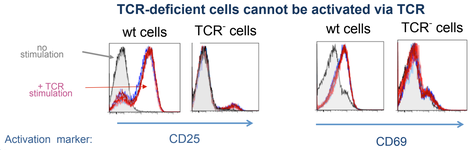


- TO IMPROVE THE THERAPEUTIC OUTCOME (COMBINATION THERAPY)



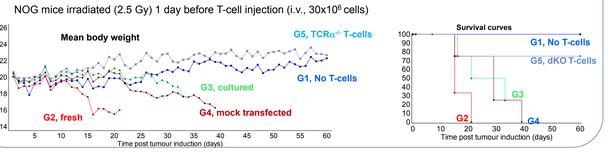
#5 Off the shelf « TCRα-deficient T-cells" for adoptive immunotherapy

TCR-deficient cells cannot be activated via TCR



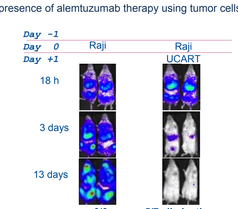
TCRα-deficient T-cells do not induce GvHD

NOG mice irradiated (2.5 Gy) 1 day before T-cell injection (i.v., 30x10^6 cells)



TCRα/CD52-deficient CAR T-cells maintain anti-tumor activity in presence of alemtuzumab

Anti-tumor activity in the presence of alemtuzumab therapy using tumor cells partially resistant to alemtuzumab (CD52$^{+/-}$ Raji-Luc cells



exposure	color range min	max
5 min	17	250
5 min	16	600
30 sec	2 000	64 000

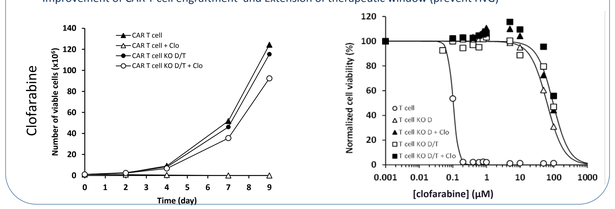
- No clinical symptoms of GvHD were observed in mice injected with CD52$^{+/-}$ TCRα-deficient T-cells. One mouse found dead at day 16 without any sign of GvHD.
- NSG mice treated with alemtuzumab combined with UCART cells or UCART alone display complete elimination of tumor cells at day 13

#6 Gene knockout of Deoxycytodine kinase (dCK) to resist to PNA

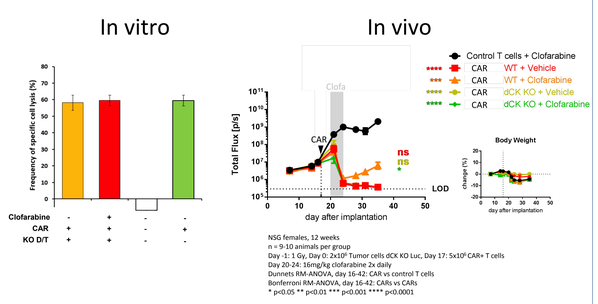
dCK-deficient CAR T-cells resist to anticancer chemotherapeutic

(Purine nucleotide analogues Clafarabine, fludarabine, cytarabine)

Improvement of CAR T cell engraftment and Extension of therapeutic window (prevent HvG)

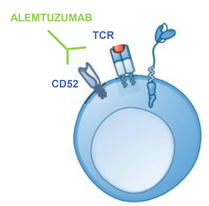


dCK-deficient CAR T-cells maintain anti-tumor activity in the presence of PNA



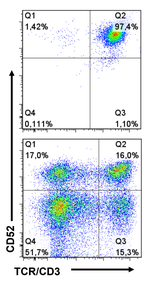
NSG females, 12 weeks
n = 9-10 animals per group
Day -1: 1 Gy, Day 0: 2x10^6 Tumor cells dCK KO Luc, Day 17: 5x10^6 CAR+ T cells
Day 20-24: 16mg/kg clofarabine 2x daily
Dunnets RM-ANOVA, day 16-42: CAR vs control T cells
Bonferroni RM-ANOVA, day 16-42: CARs vs CARs
* p<0.05 ** p<0.01 *** p<0.001 **** p<0.0001

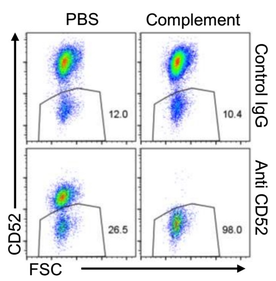
#7 TCR deficient CAR T cells Resistant to Alemtuzumab

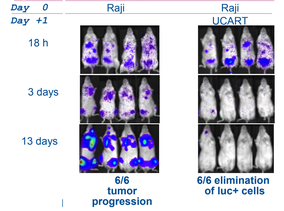


RESISTANCE TO ALEMTUZUMAB

CD52 AND TCR KO EFFICIENCY





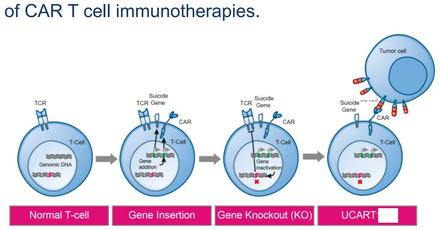


EFFICIENT DUAL INACTIVATION OF TCR AND CD52 ENABLES GENERATION OF TCR NEGATIVE CAR T CELL RESISTANT TO ALEMTUZUMAB

#8 Take Home Message

The adoptive transfer of CAR T cell represents a highly promising strategy to fight against multiple cancers. The clinical outcome of such therapies is intimately linked to the ability of effector cells to engraft, proliferate and specifically kill tumor cells within patients. When allogeneic CAR T cell infusion is considered, host versus graft and graft versus host reactions must be avoided to prevent rejection of adoptively transferred cells, host tissue damages and to elicit significant antitumoral outcome. Moreover, the ability to use CAR T cell s in Combination with currently approved drugs may provide a more efficient treatments.

We demonstrate *in vitro* and in an *in vivo* xenograft mice model, that gene editing can be used to create engineered T cells that display efficient antitumor activity and proliferate in the presence of nucleotide analogues as well as Alemtuzumab, currently used in clinic as preconditioning lymphodepleting regimens and/or antineoplastic agents. The absence of TCR at the CAR T-cell surface along with their nucleotide analogues-resistance /ALEMTUZUMAB properties could prevent their alloreactivity and enable them to be use in combination with theses reagents. By providing a basic frame work to develop a universal T cell compatible with allogeneic adoptive transfer, this work is laying the foundation stone of the large scale utilization of CAR T cell immunotherapies.



Allogenic CAR T-cells Targeting CD123 Effectively Eliminate Myeloid Leukemia Cells

Roman Galetto[1], Céline Lebuhotel[1], Alexander Bank[2], Hongliang Zong[3], Mayumi Sugita[3], Luis A. Lara-Martinez[3], Laura M. Bystrom[3], Nicole M. Cruz[3], Agnès Gouble[1], Gail J. Roboz[3], Monica L. Guzman[3] and Julianne Smith[1]

[1] Cellectis SA, Paris, France

[2] Cellectis Inc, New York, NY

[3] Division of Hematology and Oncology, Department of Medicine. Weill Cornell Medical College, New York, NY

1 Abstract

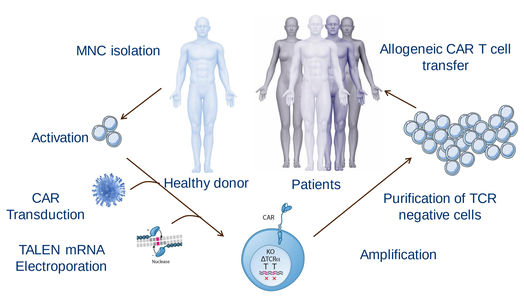
Chimeric antigen receptor (CAR)-redirected T-cells have given rise to long-term durable remissions and remarkable objective response rates in patients with refractory leukemia. At present, CAR technology is administered through the custom-made manufacturing of therapeutic products from each patient's own T-cells. However, this patient-specific autologous paradigm is a significant limiting factor in the large-scale deployment of CAR technology.

We have developed a platform for the production of "off-the-shelf" CAR T-cells from unrelated third-party donor T-cells. This platform utilizes Transcription Activator-Like Effector Nuclease (TALEN) gene editing technology to inactivate the TCRα constant (TRAC) gene, eliminating the potential for T-cells bearing alloreactive TCR's to mediate Graft versus Host Disease (GvHD). We have previously demonstrated that editing of the TRAC gene can be achieved at high frequencies, obtaining up to 80% of TCRα negative cells. This allows us to efficiently produce TCR-deficient T-cells that have been shown to no longer mediate alloreactivity in a xeno-GvHD mouse model.

Acute myeloid leukemia (AML) is incurable in the majority of patients. While allogeneic stem cell transplantation remains the most effective therapy for AML to date, other types of cellular therapy have not yet been successful in this disease. We have adapted this allogeneic platform to the production of T cells targeting CD123, the alpha chain of the interleukin-3 receptor, which is expressed in tumor cells of patients with AML. We will present both in vitro and in vivo data demonstrating specific anti-tumor activity of engineered CAR T cells against AML cells. The ability to carry out large scale manufacturing of allogeneic, non alloreactive CD123 specific T cells from a single healthy donor will offer the possibility of an off-the-shelf treatment that would be immediately available for administration to a large number of AML patients.

2 Allogeneic CAR T-cell Therapy

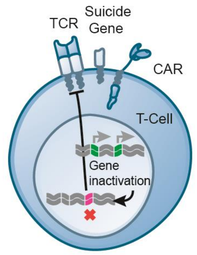
One of the key barriers to the adoptive transfer of 3rd party CAR T-cells can be overcome via the application of TALEN® gene editing technology, deletion of the TCR gene and thus the use of any donors' T-cells without the risk of GvHD.



Off-the-shelf Therapy

Cells from one donor can be used to treat multiple patients

3 UCART123

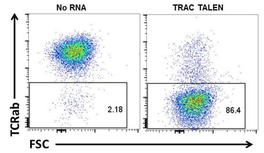


UCART123 Attributes

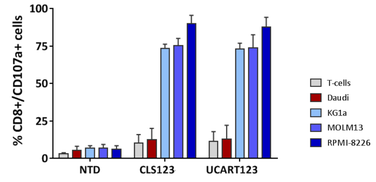
- Anti-CD123 CAR expression to redirect T cells to tumor antigens
- Suicide gene (RQR8) for safety
- TCR disruption to avoid GvHD

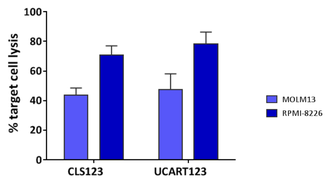
4 Activity of UCART123 cells against CD123+ cell lines

Activity of UCART123 cells (TCRαβ^{NEG}) was compared to that of non-edited T-cells expressing the same anti- CD123 CAR (CLS123).
Cells were prepared according to the following schedule:

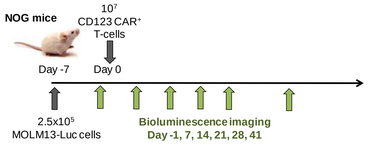


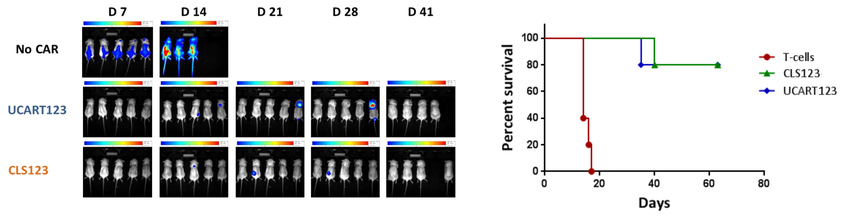
TALEN® targeting the constant region of the TCRα chain were used to disrupt TCRαβ expression for the cell surface. High levels of gene inactivation can be obtained in primary T-cells by electrotransfer of **TALEN® mRNAs** using PulseAgile electroporation.





UCART123 cells (TCRαβ^{NEG}) display equivalent in vitro activity to TCR+ T-cells expressing the same CAR molecule.

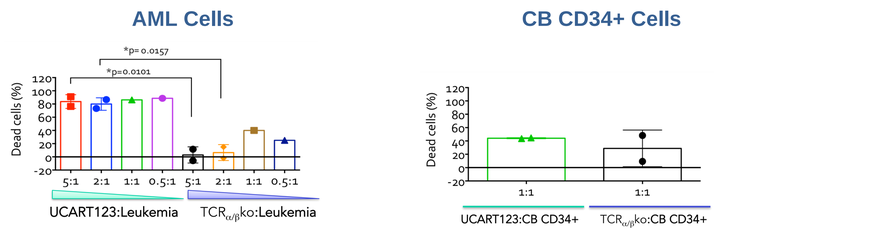




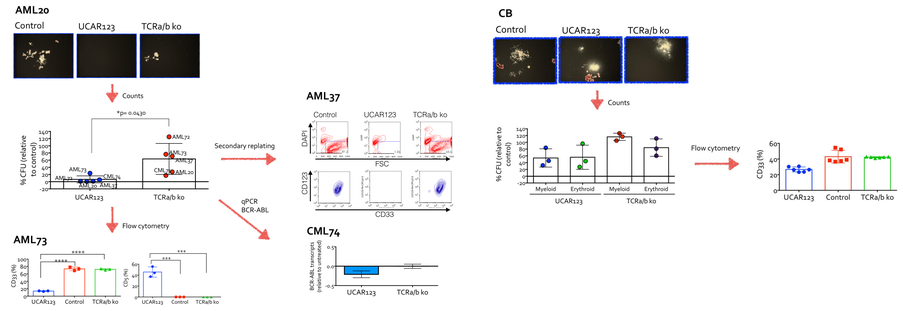
Equivalent antitumor activity was observed in mice treated with UCART123 or CLS123 T-cells.

5 UCART123 are cytotoxic to leukemia but not normal progenitor cells

UCART123 displays differential activity against AML patient cells and hematopoietic stem/progenitor cells

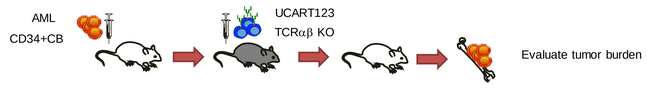


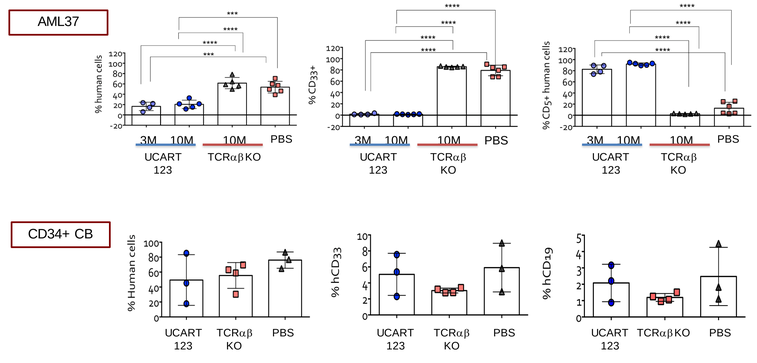
Percent dead cells was evaluated after 24 hours of exposure of primary AML or normal CB CD34+ cells to UCAR123 or TCR αβ KO T-cells.



Colony forming assays after 4 hours of exposure of CART123 or TCRαβ KO with leukemia samples or normal CD34+ CB cells.

6 UCART123 eliminate leukemia cells in AML-PDX mice





Patient derived xenografts and CD34+ CB humanized mice were treated once with 3M (AML37, CB) or 10 M (AML37) CART123 or TCRαβ KO cells. Percent human cells was determine at 2 or 3 weeks after treatment.