A Multidrug Resistant Engineered CAR T Cell for Allogeneic Combination Immunotherapy

Julien Valton[1], Alexandre Juillerat[1], Anne-Sophie Gautron[2], Philippe Duchateau[2] and Laurent Poirot[2].

[1]Cellectis Inc 430 E 29th street New York, NY 10016, [2]Cellectis SA, 8 rue de la croix Jarry 75013 Paris, France

Abstract

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 elicit successful antitumor activity in vivo. We propose to address these requirements through the development of multidrug resistant TCRαβ-deficient CAR T cells (CAR T cells KO D/T). We demonstrate that CAR T cells KO D/T are fully TCR deficient, display efficient antitumor activity and proliferate in the presence of purine nucleotide analogues (PNA), currently used in clinic as preconditioning lymphodepleting regimens. We envision that CAR T cells KO D/T could be generated from third party healthy donors and used in any patients as antitumor allogeneic immunotherapy without generating TCRαβ-dependent GvH reaction. Their drug resistance properties could enable them to resist to simultaneous infusion of lymphodepleting regimens to inhibit the host immune system and control their rate of ablation via HvG reaction.

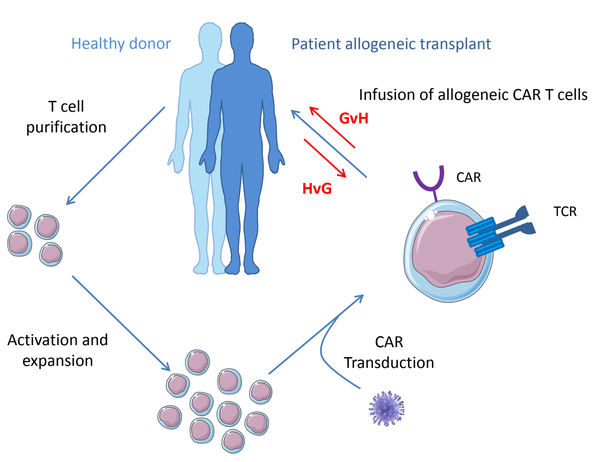


Figure 1. Principle of allogeneic CAR T cell immunotherapy illustrating graft versus host (GvH) and host versus graft (HvG) reactions that may occur after allogeneic transfer of CAR T cells

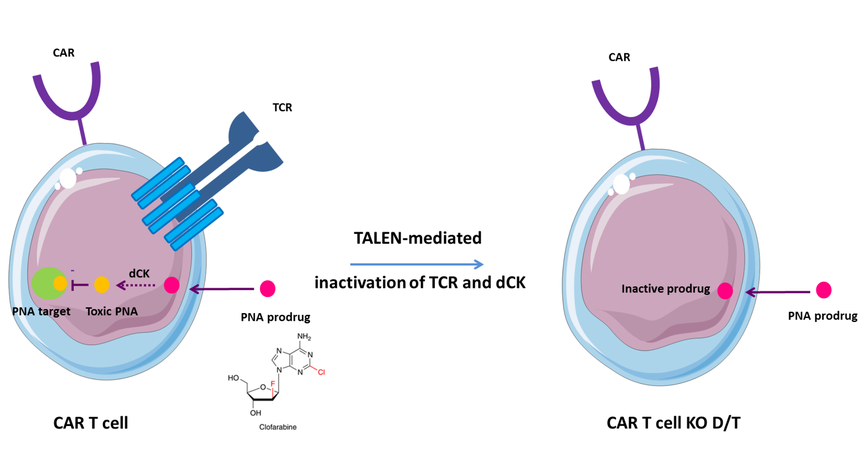


Figure 2. Genome engineering strategy to avoid HvG and GvH reactions. This strategy consists in the inactivation of TRAC and dCK (deoxycytidine kinase) genes respectively responsible for TCRαβ surface expression and purine nucleotide analogues (PNA) toxicity. Efficient depletion of TCR at the surface of CAR T cells is expect to prevent them from attacking host tissues via the TCR-dependent GvH reaction. dCK deficiency could enable them to resist to simultaneous infusion of lymphodepleting dose of PNA to inhibit the host immune system and control their rate of ablation via HvG reaction.

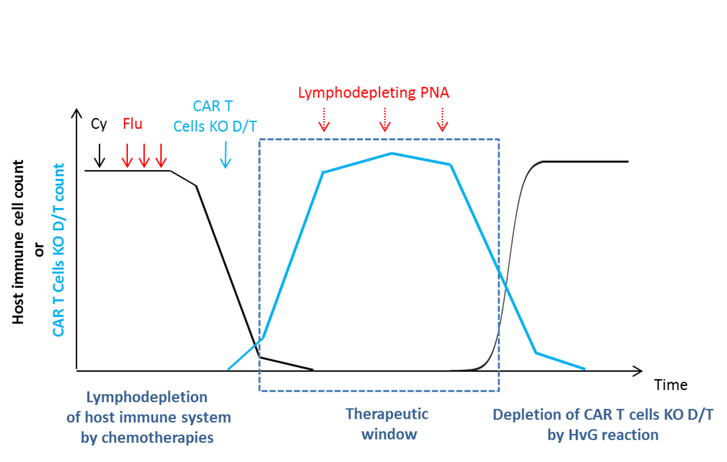
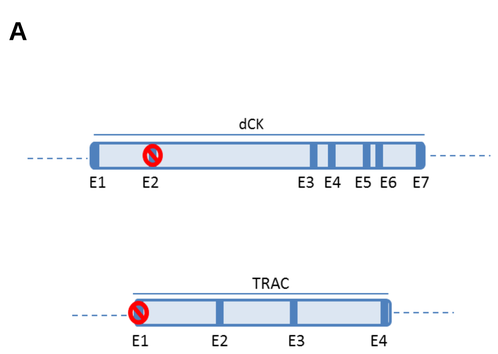


Figure 3. Schematic representation of the allogeneic adoptive transfer therapeutic window.

TALEN-mediated TRAC/dCK gene processing is efficient in primary T cells



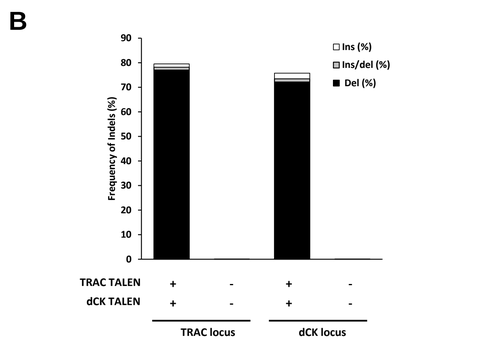
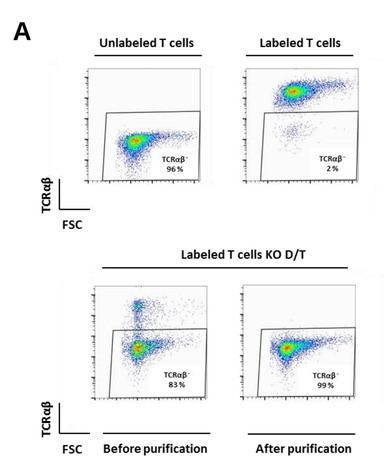


Figure 4. **TALEN treatment of primary T cells enables simultaneous and highly efficient processing of TRAC and dCK genes**. **(a)** Overall gene architectures of dCK and TRAC open reading frames. Exons (dark blue) and introns (light blue) as well as the location of TALEN-mediated gene processing (red mark) are indicated. **(b)** Genotypic characterization of TALEN-mediated inactivation of dCK and TRAC genes by high throughput DNA sequencing. T cells treated with dCK and TRAC TALEN were allowed to grow for 6 days, their genomic DNA was extracted, amplified using TRAC or dCK specific PCR amplicons and analysed by high throughput DNA sequencing. The frequencies of indels generated at TRAC and dCK locus are indicated.

TALEN-mediated TRAC gene processing disrupts TCRαβ expression at the surface of primary T cells



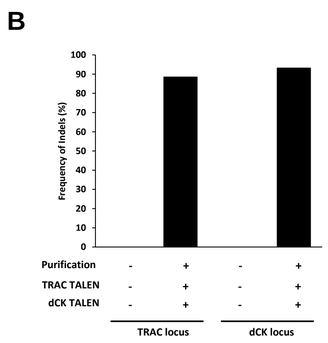


Figure 5. **Multiplex TALEN treatment of primary T cells disrupts TCR membrane expression. (a)** Purification of TRAC KO T cells from the bulk population of cell transfected with mRNA encoding TRAC and dCK TALEN. Labeling control experiment performed with T cells in the presence or in the absence of anti TCR mAb (upper panel) and monitoring of TCRαβ-deficient cells gathered before and after TRAC KO T cells purification using anti-CD3 magnetic beads and MACS LD-column (lower panel). **(b)** Genotypic characterization of TALEN-mediated inactivation of TRAC and dCK genes by high throughput DNA sequencing before and after TCR negative cells purification.

TALEN-treated CAR T cells are resistant to multiple PNAs and are able to proliferate in the presence of their clinically relevant dose

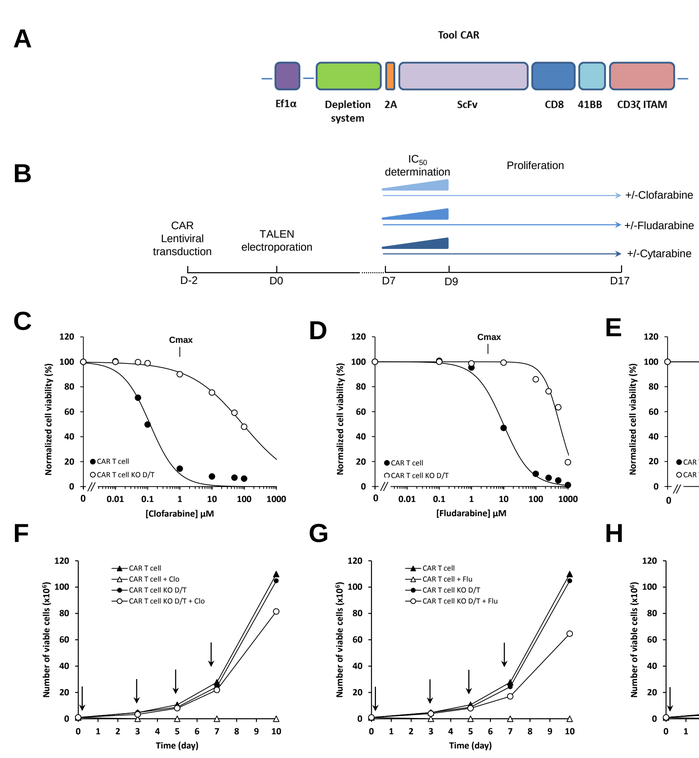


Figure 6. TALEN-treated CAR T cells are resistant to lethal dose of different purine and pyrimidine nucleotide analogues. (a) Scheme of the polycistronic DNA expression cassette encoding the membrane exposed rituximab-dependent depletion system (RQR8), followed by a 2A cis-acting hydrolase element and a tool CAR. **(b)** Scheme of the experimental workflow used to engineer and characterize T cells proliferative capacity and resistance properties toward clofarabine, fludarabine and Cytarabine. **(c, d and e)** Clofarabine, fludarabine and cytarabine IC_{50} determination. Experimental viability values obtained after 2 days of incubation were normalized with respect to the one obtained in the absence of drug. **(f, g and h).** Proliferation capacity of CAR T cells in the presence or in the absence of clofarabine, fludarabine and cytarabine. 10^6 CAR T cells were grown for a total of 10 days in the absence or in the presence of 1, 5 and 10 µM of clofarabine, fludarabine and cytarabine respectively. Vertical arrows indicate each passage step where cells were diluted to 10^6 cell/mL in the presence or in the absence of drug.

TALEN-treated CAR T cells display efficient antitumor activity

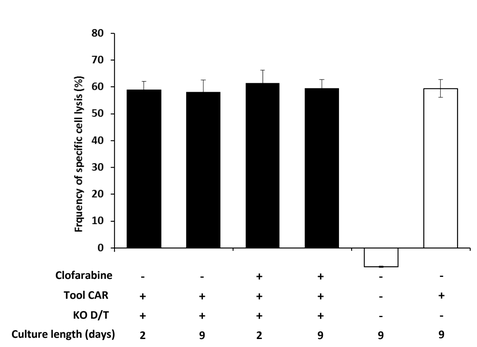


Figure 8. TALEN-treated CAR T cells retain their cytolytic activity after 2 to 9 days of culture in the presence of clinically relevant dose of clofarabine. 10^4 Daudi (target cells) and 10^4 K562 (negative control) cells labeled respectively with CellTrace™ CFSE and CellTrace™ violet were co-cultured in 100 µL for 5H at 37°C with 10^5 of TALEN-treated CAR T cells grown for 2 or 9 days in the presence or in the absence of 1 µM clofarabine. Cells were then recovered and their viability was determined by flow cytometry. Viability of Daudi and K562 cells were used to calculate the frequencies of specific cell lysis (lanes 1 to 4). T cells and CAR T cells cultured in the absence of clofarabine for 9 days were respectively used as negative and positive controls of specific cell lysis activity (lanes 5 and 6). Results are displayed as specific cell lysis activities relative to the control experiment performed with CAR T cells. Error bars represent the mean of 2 independent experiments + SD.

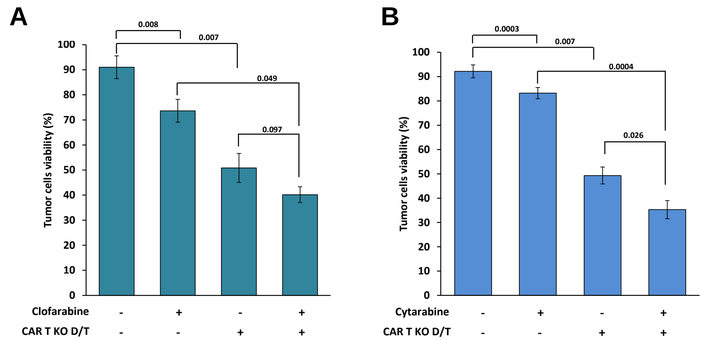


Figure 9. TALEN-treated CAR T cells can pair up with chemotherapy for better antitumor activity. 10^4 Daudi cells (target cells) preincubated in the presence or in the absence of PNA (1 µM clofarabine or 10 µM cytarabine) for 24H, were labeled with CellTrace™ CFSE and co-cultured in 100 µL for 5H at 37°C in the absence or the presence of 10^5 TALEN-treated CAR T cells (CAR T KO D/T). Cells were then recovered and their viability was determined by flow cytometry. The residual viability of tumor cells (Daudi) obtained in the presence or in the absence of clofarabine **(a)** or cytarabine **(b)** are displayed. p values numbers, calculated according to the procedure described in material and methods, are indicated. Error bars represent the mean of 2 or 3 independent experiments +/- SD performed in the presence of clofarabine or cytarabine respectively.

TALEN-treated CAR T cells can pair up with PNA chemotherapy for better antitumor activity

Conclusions

In summary, we report the development of multidrug resistant and TCRαβ-deficient CAR T cells compatible with allogeneic immunotherapy. Engineered CAR T cells resistance towards PNA chemotherapies enabled them to proliferate under lymphodepleting conditions while retaining their antitumor activity and specificity. Such PNA resistance provides two main advantages. First, it could enable allogeneically transferred CAR T cells to survive lymphodepleting regimens used to maintain the host immune system in check and control their rate of ablation via a HvG reaction. Second, it could allow combination therapy, an approach likely to improve clinical outcomes by potentializing CAR T cell antitumor activity *in vivo*. In addition, due to their lack of surface exposed-TCR, these cells could be theoretically generated from third party healthy donors and used as allogeneic immunotherapy without generating GvH reaction. In conclusion, we believe that the cellular engineering strategy described in this work will provide a basic frame work to generate a universal T cell that will foster large scale utilization of ACT and thus could benefit a broader range of patients.

Targeted genome modifications for improved adoptive T-Cell immunotherapy

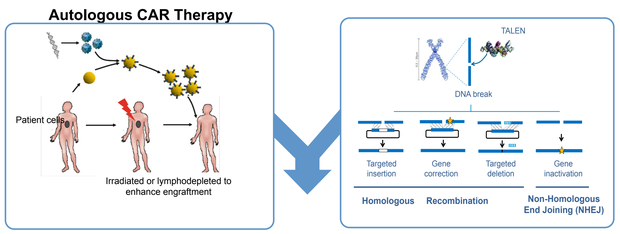
Laurent Poirot, Alexandre Juillerat, Julien Valton, Anne-Sophie Gautron and Philippe Duchateau

CELLECTIS SA, 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 while the bypass of key immune checkpoint regulators such PD1/PDL1 by inactivation of T-cells receptors would potentiate the antitumor T-cell response by impairing the interaction of the inhibitory receptor PD-1 on T-cells with PD-L1 expressed on tumor cells. Here, we will present *in vitro* and *in vivo* proof of concepts demonstrating the potential of TALEN-mediated gene editing for adoptive T-cell therapy.

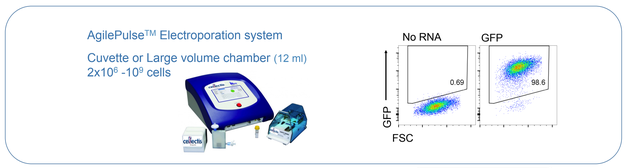
#2 Sharpening the power of T-cell by editing the genome

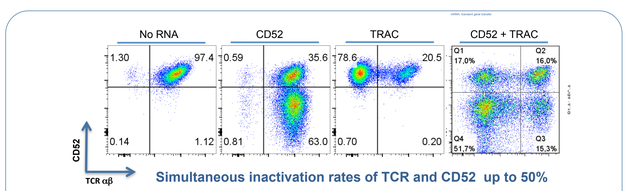


Allogeneic, non alloreactive	**Off-the-shelf product (KO TCR)**
Resistant to chemotherapy	**Compatible with SoC, Use in combination therapies (KO CD52)**
Resistant to lymphodepleting agents	**Enhanced engraftment (KO DCK)**
Resistant to tumor inhibition	**Enhanced efficacy (KO PD1, KO CTLA4,…)**
Suppressed cross T-cell reaction	**Better suited for specific tumors (T-cell auto Ag)**

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 Efficient Gene inactivation using TALEN mRNA electroporation



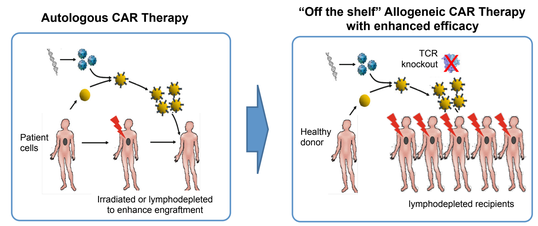


- A mean of mutation frequency ~ 80% at several loci in primary T-cells
- T-cell viability upon electroporation >90%

In order to perform gene editing, we use our proprietary PulseAgile electroporation technology to introduce nucleases as mRNA inside the target T-cell to access the cell's DNA. The mRNA molecules are rapidly degraded by the cell, which means that the nuclease is only expressed for a short time, enough to make permanent changes in the cell's targeted gene.

#4 Towards a "universal" adoptive T-cell immunotherapy

Inactivation of TCR in primary T-cells to avoid GvHD



Personalized therapy

Autologous CAR T cell therapy demonstrates impressive efficacy but faces major challenges (costs, logistics)

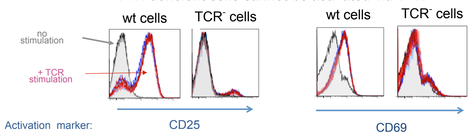
Cells from one donor could be used to treat hundreds of patients

Knocking Out the TCR gene would allow the use of allogenic CAR T-Cells without GvHD

A TALEN was designed to cleave the first exon of the TCRα constant region

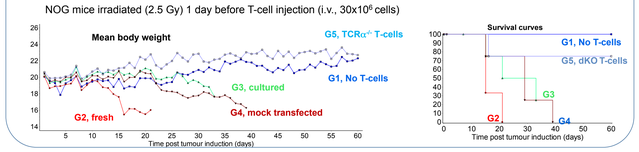
#5 Off the shelf « TCRα-deficient T-cells" for adoptive immunotherapy

TCR-deficient cells cannot be activated via TCR



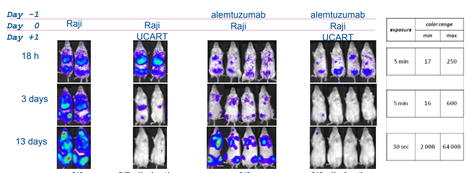
TCRα-deficient T-cells do not induce GvHD

NOG mice irradiated (2.5 Gy) 1 day before T-cell injection (i.v., 30x10⁶ 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)



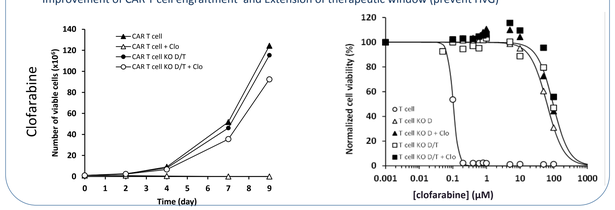
- No clinical symptoms of GvHD were observed in mice injected with CD52+/- TCRα-deficient T-cells. One mouse found dead at day 16 without any sign of GvHD.
- NSG mice treated with alemtuzumab combined with UCART cells or UCART alone display complete elimination of tumor cells at day 13

#6 T-cell engineering allows compatibility with concomitant drugs

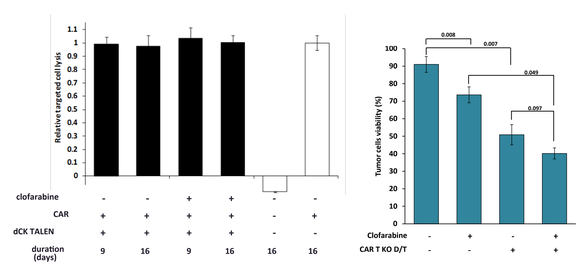
dCK-deficient CAR T-cells resist to anticancer chemotherapeutic

(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

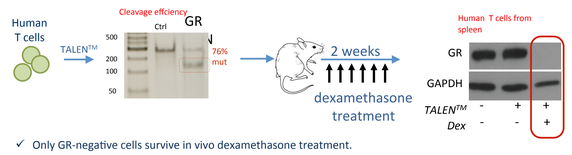


Note: TCR/dCK double KO efficiency up to 75 %

#7 Gene editing for improved antitumor CAR T-cells

Glucocorticoid Receptor-deficient T-cells resist to glucocorticoid treatment

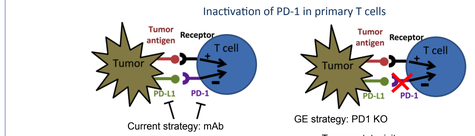
Inactivation of Glucocorticoid Receptor (GR) in primary T cells



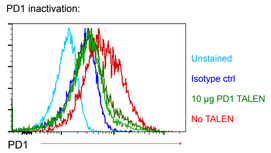
- ✓ Only GR-negative cells survive in vivo dexamethasone treatment.
- ✓ Glucocorticoid resistance opens therapeutic solutions : Prevention of allograft rejection, Control of inflammation in Glioblastoma, other brain tumors and metastasis, CMV reactivation after Graft vs Host disease

PD-1-deficient CAR T-cells have improved antitumor CAR T-cells

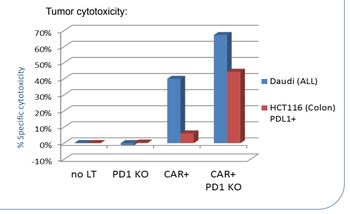
Inactivation of PD-1 in primary T cells



PD1 inactivation:



Tumor cytotoxicity:



#8 Conclusions

- Autologous CAR T-cell therapy has been very successful to cure acute Lymphoblastic Leukemia (75% CR at 12month)
- Gene editing via TALEN have the power to enhance the potential of T-cells therapy
- We have developed highly efficient TALEN platform for T-cell engineering
- TALEN modified T-cells expressing an exogenous CAR have *In vitro & in vivo* antitumor reactivity comparable to "wt" CAR T-cells
- Gene editing via TALEN have the power to alleviate some of the challenges of adoptive T-cell immunotherapy
  - ❑ Endogenous T-cell receptor → prevent GvHD → off the shelf product
  - ❑ Cellular target for a lymphodepleting treatment → proliferation and activity of T- cells in presence of immunosuppressive drugs
  - ❑ Traits can be built into T-cells to tackle tumor immune evasion and support efficient immune access to the tumor while avoiding systemic effect
  - ❑ Combined inactivation provides therapeutic solutions for indications where multiple mechanisms of escape are involved

Allogenic CAR T-cells Targeting CD123 for Adoptive Immunotherapy of Acute Myeloid Leukemia (AML)

Roman Galetto[1], Céline Lebuhotel[1], Patricia Françon[1], Agnès Gouble[1] and Julianne Smith[1]

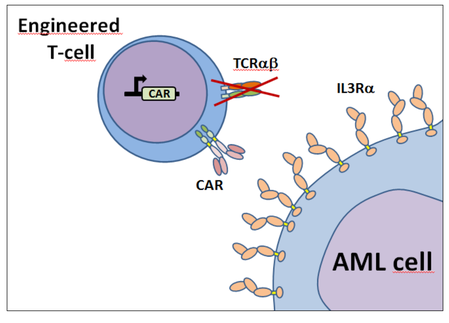
[1] Cellectis SA, Paris, France

#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. A limitation of the current autologous approach is that CAR T-cells must be manufactured on a "per patient basis". We have developed a standardized platform for manufacturing T-cells from third-party healthy donors to generate allogeneic "off-the-shelf" engineered CAR+ T-cell–based frozen products. 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. In addition to CAR expression, our T-cells are engineered to co-express the RQR8 gene as a safety feature, with the aim of rendering them sensitive to the monoclonal antibody rituximab.

In this work we present the adaptation of this allogeneic platform to the production of T cells targeting CD123, the transmembrane alpha chain of the interleukin-3 receptor, which is expressed in tumor cells from the majority of patients with Acute Myeloid Leukemia (AML). To identify an effective CAR targeting CD123, we have screened multiple antigen recognition domains in the context of several different CAR architectures to identify candidates displaying activity against cell lines expressing variable levels of the CD123 antigen. Furthermore, experiments in an AML mouse model using anti-CD123 CAR T cells demonstrate important anti-tumor activity in vivo. The ability to carry out large scale manufacturing of allogeneic, non alloreactive CD123 specific T cells from a single healthy donor will thus offer the possibility of an off-the-shelf treatment that would be immediately available for administration to a large number of AML patients.

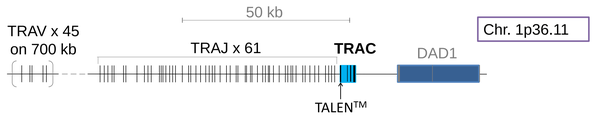
#2 Allogenic approach targeting CD123 to treat AML



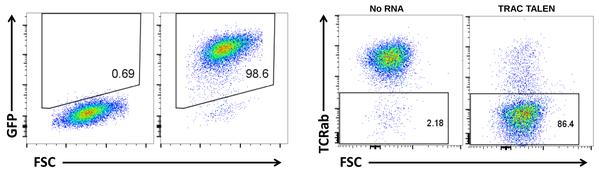
One of the key barriers to the adoptive transfer of 3rd party CAR T-cells can be overcome via the application of TALEN gene editing technology, deletion of the TCR gene and thus the use of any donors' T-cells without the risk of GvHD.

#3 TCR alpha inactivation prevents Graft vs. Host disease

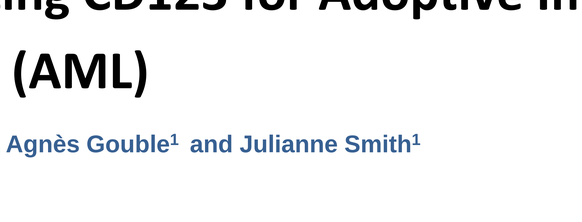
TALEN were designed to recognize and cleave the coding sequence of human TCRα gene



High levels of gene inactivation can be obtained in primary T cells by electrotransfer of TALEN mRNAs using a PulseAgile® electroporation technology.



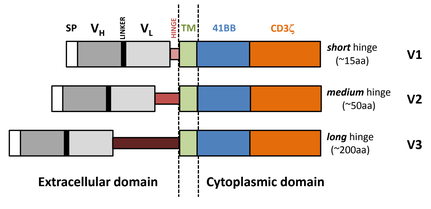
Xenogeneic model of Graft vs. Host Disease: Irradiated NOG mice were injected with PBS (group 1), or 30x10^6 human T cells from the same donor. The cells were obtained from activated PBMCs after 3 day expansion (group 2), 15-day expansion (group 3), mock electroporation and 15-day expansion (group 4), or TRAC TALEN-electroporation, 15-day expansion and depletion of TCR+ cells (group 5).



No mice receiving TRAC TALEN-treated cells showed signs of GvHD, whereas all mice receiving non engineered cells developped GvHD and had to be sacrificed. Human cells survived at least 2 weeks in all groups.

#4 Generation of anti-CD123 Chimeric Antigen Receptors

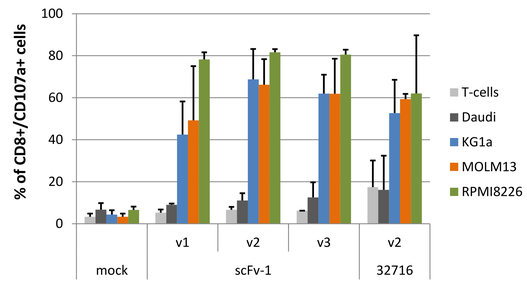
An scFv targeting the extracellular domain of the human CD123 antigen was used to construct three chimeric antigen receptors, each containing hinge sequences of different lengths.



#5 CD123 CAR T-cell in vitro activity

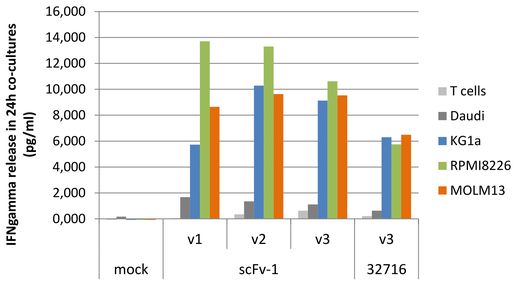
T-cells stably expressing the three CAR constructs and a benchmark CAR (32716) were generated by lentiviral transduction and their activity was assessed 10 days after transduction.

DEGRANULATION ASSAY



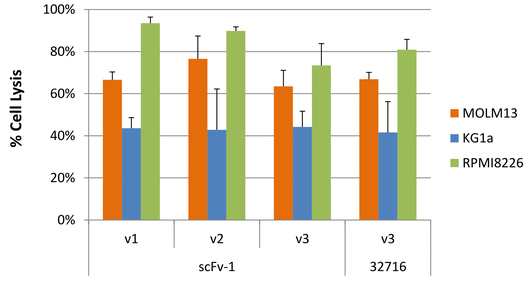
CAR+ T-cells were co-cultured with CD123+ target cells (1:1). Degranulation activity was measured by staining with CD8 and CD107a antibodies and analyzed by flow cytometry. Daudi cells were used as a negative control.

IFNgamma RELEASE ASSAY



CAR+ T-cells were co-cultured with CD123(+) target cells (1:1). The amount of IFNg released in the culture supernatants was determined by ELISA.

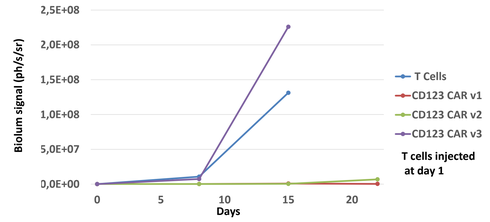
CYTOTOXICITY ASSAY



CAR+ T-cells were co-cultured with CD123(+) target cells and CD123(-) cells (10:1:1 ratio). The viability of each cell line -CD123(+) and CD123(-)- was determined after 4h of co-culture to determine the % of specific cell lysis.

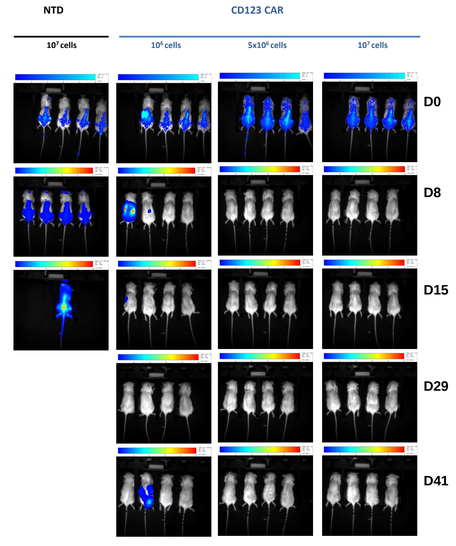
#6 CD123 CAR T cell in vivo anti-tumor activity

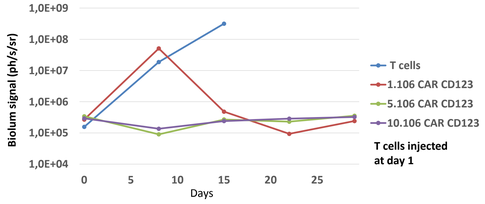
All three CAR constructs were evaluated for vivo anti-tumor activity: 5×10^6 CAR-positive T-cells were intravenously injected in NSG mice 7 days after tumor cell injection (2.5×10^5 Molm13-ffluc cells).



The CD123 CAR v1 and v2 display a strong anti-tumor effect while the CD123 CAR v3, despite significant in vitro activity, did not display any anti-tumor activity in vivo.

The anti-tumor activity of the v2 CAR was further characterized in a long term anti-tumor study: several doses of CAR-positive T-cells (1×10^6, 5×10^6 and 10×10^6 cells) were intravenously injected in NSG mice 7 days after tumor cell injection (2.5×10^5 Molm13-ffluc cells) and mice were followed for up to 60 days. The T cells were injected at Day 1 in the figures below.





Except for one mouse (treated at 1x10^6), all the mice treated with CAR T-cells were alive at Day 60. In addition, flow cytometry analysis at Day 60 confirmed the complete elimination of the tumor cells in the blood, the spleen and the bone marrow.