

Sam Cullison

Synthetic Biology

## Cell Hacker Final Report

### Background

CAR T cell therapies are created through engineering a donor's T cells to fight cancer. In general, the CAR (Chimeric Antigen Receptor) enables the T cell to selectively eliminate a target cell (normally a tumor) using an antibodies' scFv coupled to an intracellular signaling domain. Advantages of CAR therapies include no required immunosuppression, the long-lasting treatment life, and the limitation of off target effects. The ingenuity and modularity of the CAR therapy design has led to rapid innovation, and there are over one thousand clinical trials currently ongoing for different potential therapies<sup>1</sup>. Due to the illusive and ever-mutating characteristics of cancer, selecting a CAR's target antigen is extremely difficult. However, by specifying the target (and non-target) antigen(s), the following report simplifies therapy development significantly, and offers a great introduction into the design and application of CAR T cell therapies.

### Problem Statement

The Cell Hacker project proposed to my group challenged us to build a CAR T cell therapy which used NOT-gated logic to selectively target tumor cells and limit off-target effects. To differentiate the tumor and non-tumor cells, the tumor cells were specified to be Axl<sup>+</sup> while the non-tumor cells to be avoided were HER2<sup>+</sup>. Additionally, the engineered T cells were to promote IL-12 expression in response to engagement with the Axl antigen. Once this therapy was created, we contrasted it against alternative designs and benchmarked its performance through a series of experiments. Specifically, we designed experiments to evaluate a) our logic gates stringency/discriminatory power b) the cytotoxicity of our engineered T-cells c) the phenotype of our cells after exposure to Axl<sup>+</sup> cells and d) the effectiveness of the IL-12 gene circuit in response to Axl<sup>+</sup> cells.

### Solution

A CAR is comprised of a recognition domain, transmembrane domain, and signaling domain. For a singular CAR, the recognition domain determines the input (being the ligand of the domain's scFv), and the output is the intracellular signaling pathway of the protein which makes up the signaling domain<sup>2</sup>. Due to this singular input-output relationship, one CAR is not enough to construct a NOT-gate. Various CAR designs allow NOT-gated logic, but the iCAR/aCAR was selected for this project. The iCAR/aCAR system is comprised of an inhibitory CAR (iCAR) and an activating CAR (aCAR) which together provide the NOT-gated activation of the T cells<sup>3</sup>. The aCAR's scFv recognizes the T cell's activating stimulus and, once bound, enzymes on the signaling domain activate the T cell through endogenous signaling pathways. The proposed solution's aCAR features an anti-Axl scFv and a signaling domain comprised of CD3 $\zeta$  and CD28. Similar to the aCAR in construction, but opposite in response is the iCAR. The iCAR recognizes the desired inhibitory stimulus of the T cell and reverses/prevents activation, thus counteracting the effects of the aCAR. The iCAR of the proposed solution includes an anti-HER2 scFv a signaling domain comprised of PD-1. A diagram showing the system is presented below (Figure 1)

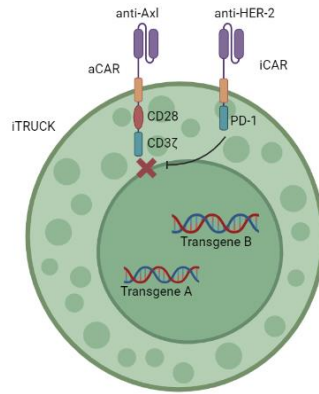


Figure 1: The Solution

The specific mechanism of the proposed aCAR/iCAR system relies on endogenous pathways which activate and inhibit T cell activity. For the activating CAR, upon Axl recognition, three immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic domain of the CD3 $\zeta$  chain are phosphorylated by the kinase Lck<sup>4</sup>. The phosphorylated ITAMS and Lck then initiate a cascade of signaling events which lead to the transcription of factors such as NF- $\kappa$ B, AP-1, and NFAT, and expression of genes involved in T cell proliferation, cytokine production, and cytotoxicity<sup>5</sup>. CD3 $\zeta$  alone does not elicit a competent enough activation signal<sup>6</sup>, so the costimulatory signal CD28 is present on the signaling domain of the aCAR as well. CD28, or specifically the cytosolic domain of CD28, has two effects once activated. First, it interacts with CD3 $\zeta$  to further promote the phosphorylation of molecules in the CD3 $\zeta$  signaling pathway<sup>7</sup>. This effect leads to greater expression of NF- $\kappa$ B and NFAT and greater T cell activation. Secondly, CD28 increases the expression of other costimulatory domains, for example CD40L and OX40, and these other domains further promote activation of the T cell (along with apoptosis evasion and T cell survival). Overall, combination of CD28 and CD3 $\zeta$  in the aCAR's signaling domain leads to T cell activation upon Axl<sup>+</sup> presence. Inversely related is the iCARs PD-1, which inhibits the activating effects of the aCAR. When PD-1 is activated, it recruits SHP-2 and proceeds to dephosphorylate CD3 $\zeta$ , CD28, and various molecules in the CD28 pathway<sup>8</sup>. A diagram is presented below which illustrates the molecules involved (Figure 2<sup>5</sup>), but in the end T cell proliferation, cytokine production, and proliferation are inhibited.

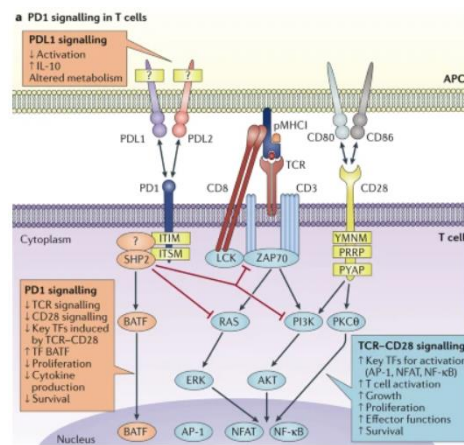


Figure 2 (Sharpe et. Al)

The aCAR/iCAR design explained above offers several advantages over alternative methods, the first of which is the reversibility of the T cell function. Research has found that aCAR/iCAR yields highly reversible effects<sup>3</sup>, meaning that the T cells maintain functionality and are not permanently exhausted after contact with the inhibitory stimulus. Reversibility is critical to maintaining long term efficacy and viability of CAR T cell therapy treatment. Other logic designs, such as those using syn-Notch, permanently disable the T cell after inhibitory stimulus<sup>9</sup>. Another advantage offered by the aCAR/iCAR NOT-gated design is high discriminatory power. The original aCAR/iCAR paper reported a 95% reduction in cytotoxicity through use of a PD-1 iCAR<sup>3</sup>. Although this 95% reduction was gathered at low effector to target(E/T) ratios, high E/T ratios yielded ~60% cytotoxicity reduction. Research using another alternative, zipFVs, reported much lower and less significant inhibitory responses (cytotoxicity reduction of ~ 50%)<sup>10</sup>. Finally, PD-1 provides greater inhibition than alternatives like CTLA-4. In a study using a CTLA-4 iCAR and a PD-1 iCAR, the cytokine reduction reported was 78-88% and 55-71% respectively<sup>3</sup>. This all goes to show that the aCAR/iCAR design endows beneficial therapy properties, such as reversibility and effectiveness, which are not offered by alternative NOT-gate approaches.

The final part of our CAR T therapy includes two transgenes. The first, designated Transgene A, contains the constitutive promoter EF1 $\alpha$  and the genes which encode for the aCar, iCar, and GFP (later used for validation of transfection). A ribosome skipping sequence is located in-between each of these genes. Transgene B contains an inducible NFAT-RE-IL-2 promotor<sup>11</sup> followed by the genes encoding for IL-12 and BFP. A ribosomal sequence separates the IL-12 and BFP genes as well. The NFAT activated promoter for transgene 2 was selected due to the signaling pathways of the aCAR. Previously mentioned, CD28 and CD3Z promote NFAT expression upon activation, and thus link the presence of Axl to the expression of IL12. With the presence of an inhibitory stimulus, PD-1 signaling will repress expression of NFAT and thus IL12. Repression of IL12 expression upon inhibitory stimulus is characteristic of a good gene circuit design, because uninhibited IL12 expression of T cells could lead to health issues and possibly cancer<sup>12</sup>.

## Methods

The first step in implementing our solution involves the placement of the transgenes into the nucleus of our donor T cells. To do this transfection, we will use synthetic DNA nanocarriers<sup>13</sup>. Synthetic DNA nanocarriers facilitate the selective transfection (Figure 3) of circulating T cells in-situ through anti-CD3e fragment covered biodegradable nanoparticles. These nanoparticles bind to the plasma membrane of CD3+ T cells and are subsequently transported into the cytosol via receptor-mediated endocytosis. The nanocarriers are functionalized with microtubule-associated sequences (MTAS) and nuclear localization signals (NLS), which enable nuclear import of the nanocarrier's genetic cargo. Once in the nucleus piggyBac transposons, which flank transgenes A and B on their respective plasmids, integrate the transgenes into the chromosomes. This integration occurs through a cut and paste mechanism and is mediated through a piggyBac transposase enzyme (which is encoded for by another plasmid present in the nanocarrier).

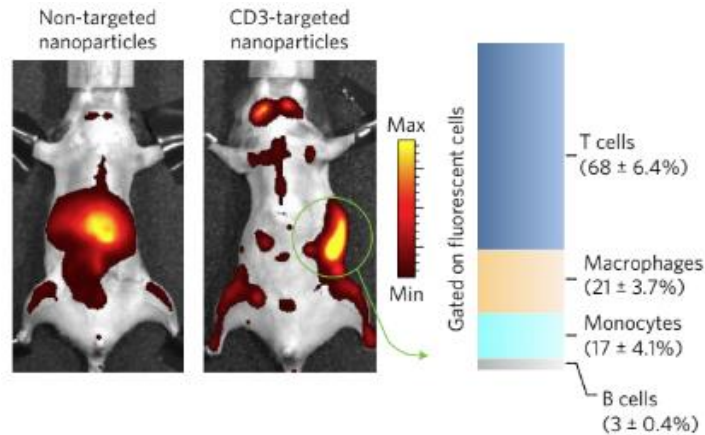


Figure 3: The Specificity and discrimination of the synthetic DNA nanocarriers (Smith et. al)

To assess the proposed transfection, T cells will be extracted from a donor and cultured with the nanoparticles in vitro. After culturing for 24 hours, the T cells will be analyzed through a fluorescence assay. The presence of GFP (located downstream of aCAR and iCAR on the transgene) will validate the transfection. Once in-vitro success is established, mouse models will be transfected to assess any global off-target effects of the transfection. For this evaluation, first the mice will be transfected with the active nanocarrier. 12 hours later mice tissue of interest will be removed and analyzed by both cell type and fluorescence. Testing by cell type and fluorescence allows verification of the presence of the iCAR/aCAR system and quantification of the nanocarrier's discrimination of non-T cells.

There are several benefits to using nanoparticles to globally deliver CAR therapies. First, nanoparticles are easy to manufacture and store which leads to lower costs<sup>13</sup>. Second, with nanocarriers there is no need to isolate T cells from a patient, engineer them with complex viral transduction systems, and reintroduce the therapy (which is the usual, time and cost intensive delivery process)<sup>14</sup>. Skipping these steps allows for greater scalability, and reduced costs. Despite these advantages, a major downside to the nanoparticle delivery system is the lack of FDA approval. Unlike lentiviral and retroviral transduction, which have cleared clinical trials, synthetic DNA nanoparticles have yet to pass and be deemed effective by the FDA (although numerous clinical trials using similar nanoparticles are currently ongoing).

## Evaluating Performance

To evaluate the cytotoxicity of our T cells we will use CyQuant cytotoxicity assays, which work by measuring the amount of LDH released by dead/dying cells over time. After establishing a baseline, these cytotoxicity assays report a percent change in cytotoxicity between the baseline and the culture of interest. The first experiment will analyze the cytotoxicity of the engineered T cells in the presence of only target cells. A sample of engineered T cells will be placed into a culture containing only Axl+ tumor cells, and a separate sample of only Axl+ tumor cells (no T cells present) will serve as a control. After 12 hours the CyQuant assay will be conducted on each plate, and the difference between the results will represent the target cells eliminated by the T cells.

The next cytotoxicity assay will evaluate the discriminatory power of the aCAR/iCAR system. Engineered T cells will be cultured in 4 plates which have either Axl+HER2-, Axl-

HER2+, Axl+HER2+, or Axl-HER2- cells<sup>3</sup>. After 12 hours each culture will be analyzed through both the CyQuant assay, and a fluorescence assay. The cytotoxicity assay will show the discriminatory power of the system, and ideally it would reveal a significant peak in the Axl+, HER2- culture and 3 smaller peaks for the other dishes (with these three peaks being caused by natural degradation/cell death). Fluorescence assays will also be conducted on each culture to measure T cell IL-12 production (evidenced by the amount of BFP expression). IL-12 production serves as another indicator of T cell activation due to its promotion by the activating, Axl binding, CAR. Additionally, the IL-12 BFP test would validate the expression of IL-12 upon activation.

The reversibility of the engineered T cells will be evaluated next. As stated before, the reversibility of the inhibitory mechanism is critical to effective and long-lasting therapy, and it will be evaluated through flow cytometry and fluorescence assays. Culture 1 is defined as CAR T cells with strictly target cells, and culture 2 as CAR T cells with strictly non-target cells. After 48 hours both cultures will undergo flow cytometry, which serves to isolate the GFP-producing T cells from the non/target cells in the plate. After this, the T cells from culture 1 and 2 will separately be cultured with only target cells. After another 48 hours, both cultures will undergo cytotoxicity assays. Culture 1's T cell's cytotoxicity (T cells that went from target -> target) will be designated as Meas1, and culture 2's T cell's cytotoxicity (T cells went from non-target -> target) as Meas2. The difference between Meas1 and Meas2 indicates a critical aspect of the reversibility of the engineered T cells, for it shows the effect of the inhibitory antigen on the T cells functionality<sup>3</sup>. If the measurements are close to one another, it means that the inhibitory pathway was indeed reversible, and non-target cells did not significantly affect the T cells response to target cells. If the measurements are not close then the T cell's functionality was affected by the inhibitory antigen, and the therapy design needs to be changed.

Finally, flow cytometry will be characterize T cell phenotype after exposure to Axl. T cells will be exposed to Axl+ target cells and incubated with fluorescent antibodies which tag for CD45RO, and PD-1. The antibody for CD45RO will label the T cells that form a memory compartment, and the PD-1 marker will label those that are exhausted. When T cells become exhausted, they lose their functionality and are not nearly as effective at responding to targets<sup>15</sup>. If the proposed CAR T cells become readily exhausted after activation by Axl+ targets, it will minimize the effectiveness of the therapy and require an increase in the frequency of treatment (repeated doses). To further examine the possible exhaustion of our T cells, the phenotype will also be characterized after exposure to strictly non-target (HER2+) cells. Although PD-1 is a key marker of exhaustion in T cells, it is also used in the iCAR signaling domain. To limit potential cross talk between the inhibitory signal and endogenous PD-1L, PD-1 and LAG3 fluorescent antibodies will both be used to evaluate T cell exhaustion<sup>16</sup>. After testing, if a high amount of permanent T cell exhaustion follows inhibitory stimulation, it means the engineered T cells exhibit poor reversibility. Extreme amounts of exhaustion could also mean that the iCAR's PD-1 signaling pathway exhibits crosstalk with the T cells endogenous PD-1/PD-1L expression. Specifically, exogenous PD-1 signaling could promote endogenous PD-1L and PD-1 expression which, when overexpressed, are causes of T cell exhaustion<sup>15</sup>. If this were true, a different protein for the iCAR signaling domain would be selected to provide more controlled and reliable inhibition.

## Discussion

CAR T cell therapies provide a wide range of solutions to intricate and complex problems. The NOT-gated aCAR/iCAR therapy discussed above is one of several viable solutions that lead to discriminate targeting of Axl+, HER2- tumor cells, and release of IL12 upon activation. However, improvements to the proposed design can be made in the future. For one, using multiple inhibitory signaling proteins on the iCAR can increase the T cells discriminatory power. CTLA-4 or BTLA are two proteins to consider adding. And second, by separating the expression of the iCAR and aCAR, the off target affects and reversibility of the T cell can be altered. Separation of expression would require different promoters, but by evaluating the performance of different ratios, the aCAR/iCAR system could be further optimized to match desired criteria.

## References

- (1) Wang, V.; Gauthier, M.; Decot, V.; Reppel, L.; Bensoussan, D. Systematic Review on CAR-T Cell Clinical Trials Up to 2022: Academic Center Input. *Cancers* **2023**, *15* (4), 1003. <https://doi.org/10.3390/cancers15041003>.
- (2) Lim, W. A.; June, C. H. The Principles of Engineering Immune Cells to Treat Cancer. *Cell* **2017**, *168* (4), 724–740. <https://doi.org/10.1016/j.cell.2017.01.016>.
- (3) Fedorov, V. D.; Themeli, M.; Sadelain, M. PD-1– and CTLA-4–Based Inhibitory Chimeric Antigen Receptors (iCARs) Divert Off-Target Immunotherapy Responses. *Sci. Transl. Med.* **2013**, *5* (215). <https://doi.org/10.1126/scitranslmed.3006597>.
- (4) Ying, Z.; He, T.; Wang, X.; Zheng, W.; Lin, N.; Tu, M.; Xie, Y.; Ping, L.; Zhang, C.; Liu, W.; Deng, L.; Qi, F.; Ding, Y.; Lu, X.; Song, Y.; Zhu, J. Parallel Comparison of 4-1BB or CD28 Co-Stimulated CD19-Targeted CAR-T Cells for B Cell Non-Hodgkin's Lymphoma. *Mol. Ther. - Oncolytics* **2019**, *15*, 60–68. <https://doi.org/10.1016/j.omto.2019.08.002>.
- (5) Sharpe, A. H.; Pauken, K. E. The Diverse Functions of the PD1 Inhibitory Pathway. *Nat. Rev. Immunol.* **2018**, *18* (3), 153–167. <https://doi.org/10.1038/nri.2017.108>.
- (6) Weinkove, R.; George, P.; Dasyam, N.; McLellan, A. D. Selecting Costimulatory Domains for Chimeric Antigen Receptors: Functional and Clinical Considerations. *Clin. Transl. Immunol.* **2019**, *8* (5), e1049. <https://doi.org/10.1002/cti2.1049>.
- (7) Esensten, J. H.; Helou, Y. A.; Chopra, G.; Weiss, A.; Bluestone, J. A. CD28 Costimulation: From Mechanism to Therapy. *Immunity* **2016**, *44* (5), 973–988. <https://doi.org/10.1016/j.immuni.2016.04.020>.
- (8) Xu, X.; Hou, B.; Fulzele, A.; Masubuchi, T.; Zhao, Y.; Wu, Z.; Hu, Y.; Jiang, Y.; Ma, Y.; Wang, H.; Bennett, E. J.; Fu, G.; Hui, E. PD-1 and BTLA Regulate T Cell Signaling Differentially and Only Partially through SHP1 and SHP2. *J. Cell Biol.* **2020**, *219* (6), e201905085. <https://doi.org/10.1083/jcb.201905085>.
- (9) Choe, J. H.; Watchmaker, P. B.; Simic, M. S.; Gilbert, R. D.; Li, A. W.; Krasnow, N. A.; Downey, K. M.; Yu, W.; Carrera, D. A.; Celli, A.; Cho, J.; Briones, J. D.; Duecker, J. M.; Goretsky, Y. E.; Dannenfelser, R.; Cardarelli, L.; Troyanskaya, O.; Sidhu, S. S.; Roybal, K. T.; Okada, H.; Lim, W. A. SynNotch-CAR T Cells Overcome Challenges of Specificity, Heterogeneity, and Persistence in Treating Glioblastoma. *Sci. Transl. Med.* **2021**, *13* (591), eabe7378. <https://doi.org/10.1126/scitranslmed.abe7378>.

- (10) Cho, J. H.; Okuma, A.; Sofjan, K.; Lee, S.; Collins, J. J.; Wong, W. W. Engineering Advanced Logic and Distributed Computing in Human CAR Immune Cells. *Nat. Commun.* **2021**, *12* (1), 792. <https://doi.org/10.1038/s41467-021-21078-7>.
- (11) Chmielewski, M.; Abken, H. TRUCKS, the Fourth-generation CAR T Cells: Current Developments and Clinical Translation. *Adv. CELL GENE Ther.* **2020**, *3* (3). <https://doi.org/10.1002/acg2.84>.
- (12) Jia, Z.; Ragoonanan, D.; Mahadeo, K. M.; Gill, J.; Gorlick, R.; Shpal, E.; Li, S. IL12 Immune Therapy Clinical Trial Review: Novel Strategies for Avoiding CRS-Associated Cytokines. *Front. Immunol.* **2022**, *13*, 952231. <https://doi.org/10.3389/fimmu.2022.952231>.
- (13) Smith, T. T.; Stephan, S. B.; Moffett, H. F.; McKnight, L. E.; Ji, W.; Reiman, D.; Bonagofski, E.; Wohlfahrt, M. E.; Pillai, S. P. S.; Stephan, M. T. In Situ Programming of Leukaemia-Specific T Cells Using Synthetic DNA Nanocarriers. *Nat. Nanotechnol.* **2017**, *12* (8), 813–820. <https://doi.org/10.1038/nnano.2017.57>.
- (14) Parayath, N. N.; Stephan, S. B.; Koehne, A. L.; Nelson, P. S.; Stephan, M. T. In Vitro-Transcribed Antigen Receptor mRNA Nanocarriers for Transient Expression in Circulating T Cells in Vivo. *Nat. Commun.* **2020**, *11* (1), 6080. <https://doi.org/10.1038/s41467-020-19486-2>.
- (15) Lee, J.; Ahn, E.; Kissick, H. T.; Ahmed, R. Reinvigorating Exhausted T Cells by Blockade of the PD-1 Pathway. *Forum Immunopathol. Dis. Ther.* **2015**, *6* (1–2), 7–17. <https://doi.org/10.1615/ForumImmunDisTher.2015014188>.
- (16) Yi, J. S.; Cox, M. A.; Zajac, A. J. T-cell Exhaustion: Characteristics, Causes and Conversion. *Immunology* **2010**, *129* (4), 474–481. <https://doi.org/10.1111/j.1365-2567.2010.03255.x>.