Presented on Wednesday, May 11, 8:00 AM

960 - **Precise Tumor Targeting with NOT Logic-Gated Chimeric Antigen Receptor Gene Circuits**

Author Block: **Nicholas W. Frankel*, Seunghee Lee*,** Derrick Lee, Marcus Gainer, Brian S. Garrison, Travis Wood, Wesley Gorman, Russell Gordley, Marcela Guzman Ayala, Tim Lu, Gary Lee, Wilson Wong (*equal contribution)

Disclosure Block: **N.W. Frankel:** 1; Commercial Interest *i.e. Company X*; Senti Biosciences. 1; What was received? *i.e. Honorarium*; I am an employee of Senti Biosciences, and receive salary and benefit from the company. 1; For what role? *i.e. Speaker*; Researcher.

Background: The engineering of chimeric antigen receptors (CARs) into T cells or NK cells can redirect them to kill cancer cells that express a specific surface antigen. This technology has led to breakthrough therapies for cancer, but the lack of uniquely cancer-specific antigens has the risk of life-threatening on-target, off-tumor toxicity. CAR-engineered immune cells could be used to treat a broader spectrum of cancers if recognition of a “safety antigen” on healthy cells could selectively block killing. Here, we engineered T and NK cells with a synthetic NOT logic-gated gene circuit: an activating CAR (aCAR) drives killing of targets presenting an activating antigen, while an inhibitory CAR (iCAR) suppresses cytotoxicity against targets expressing a safety antigen (SA).

Methods: iCARs consisted of an SA-binding domain, hinge and transmembrane domains, and a functional intracellular domain (ICD) derived from the cytoplasmic tails of inhibitory coreceptors containing immunoreceptor tyrosine-based inhibitory motifs. We first constructed a panel of iCARs with different ICDs and performed initial screens for expression and function in T cells. We then developed protocols for transducing aCAR/iCAR pairs in NK cells. Finally, we validated the performance of top aCAR/iCAR combinations in NK cells by measuring cytotoxicity and cytokine release in response to tumor cells that expressed activating antigen +/- SA. AML, logic-gated CAR-NK cell technology has applicability to other cancerassociated antigens limited by potential off-tumor toxicity.

Results: NOT-gated NK cells with an iCAR inhibiting a second generation aCAR with a CD28 costimulatory domain significantly reduced both cytotoxicity (32%, $p<0.05$) and secretion of TNFa, IFNg, and Granzyme B (50%, 54%, and 18%, respectively; $p<0.05$ for all differences) in response to SA+ targets, while aCAR-only NK cells exhibited no such significant differences. The NOT logic gate gene circuit did not interfere with aCAR performance since, in the absence of SA on target cells, NOT-gated CAR NK cells showed no reduction in killing performance or cytokine release compared to “conventional” aCAR-only NK cells. When SA+ and SA- target cells were mixed, NOT-gated CAR-NK cells preferentially killed SA- target cells over SA+ target cells, while aCAR-only NK cells killed both subpopulations equally. These data show that this NOT gate gene circuit enables safer killing of SA- target cells, discriminating them from SA+ target cells on a cell-by-cell basis.

Conclusion: We have developed a best-in-class NOT gate gene circuit technology and demonstrated it in therapeutic CAR-T and CAR-NK cells. NK cells can be used in an allogeneic fashion and can reduce cost and increase patient access relative to autologous products. Therapeutic cells with these NOT-gated CAR gene circuits may improve safety for currently validated cancer antigens, and also enable new therapies for previously unaddressed indications.

Precise Tumor Targeting with NOT GATE Chimeric Antigen Receptor Gene Circuits

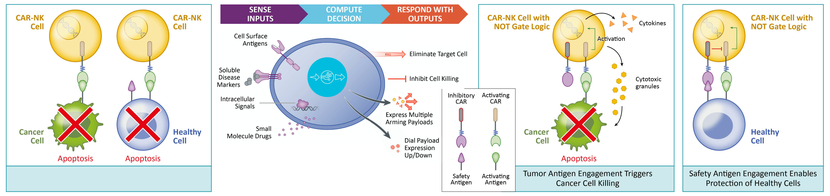
ASGCT 2021
Abstract #960

Nicholas W. Frankel[1]*, Seunghee Lee[2]*, Derrick Lee[1], Marcus Gainer[1], Brian S. Garrison[1], Travis Wood[1], Wesley Gorman[1], Russell Gordley[1], Marcela Guzman Ayala[1], Tim Lu[1], Gary Lee[1], Wilson Wong[2] (*equal contribution)

[1]Senti Biosciences, Inc. South San Francisco, CA
[2]Boston University Boston, MA

NOT GATE can prevent on-target/off-tissue toxicities of CAR immune cells

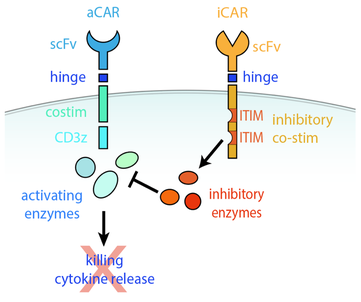
1. Current CAR-based cell therapies cannot distinguish between cancer and healthy cells, causing on-target/off-tissue toxicities.
2. Senti's logic gate gene circuits can protect healthy tissues and cells from CAR-mediated cytotoxicity.
3. Senti's NOT GATE CAR-NK cells can enable tumor cell killing while sparing healthy cells.



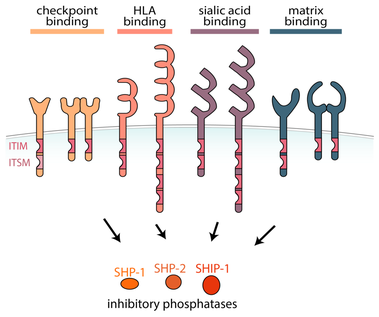
Engineering smarter medicines by using logic-gated CAR-NK cells which can provide selective targeting and avoid on-target/off-tissue toxicities enabling precise targeting of tumor antigens. Senti has developed a NOT GATE that can provide selective targeting of tumor cells while protecting healthy cells in an antigen-dependent manner. Senti's proprietary gene circuits allow cells to recognize tumor antigens as well as Safety Antigens present only in healthy cells. Cells will then carry out defined functions. In this case, the use of a NOT GATE allows for anti-gen-mediated tumor cell killing with decreased cytotoxicity and cytokine production in a Safety Antigen dependent manner. Here we present the proof-of-concept of NOT GATE functionality in NK cells in which a tumor-targeting activating CAR (aCAR) is paired with an inhibitory CAR (iCAR) that recognizes a Safety Antigen in normal cells. Multiple naïve inhibitory domains have been tested for iCARs to select those that resulted in robust antigen-mediated inhibition of NK cell cytotoxicity. NOT GATE gene circuits were recently published by Senti's scientific advisor and collaborator Dr. Wilson Wong, Boston University [Cho, JH, et al., Nat Comm 2021].

Strategy for engineering iCAR-based NOT GATE circuits in CAR-immune cells

General inhibitory CAR structure and function



Native inhibitory receptors under testing as iCARs



Intracellular domain (ICD) screening and validation methodology

Screen families of inhibitory ICDs using characteristic examples

ICD 1
ICD 2
ICD 3
ICD 4
ICD 5

Explore other members of receptor families with iCAR-compatible ICDs

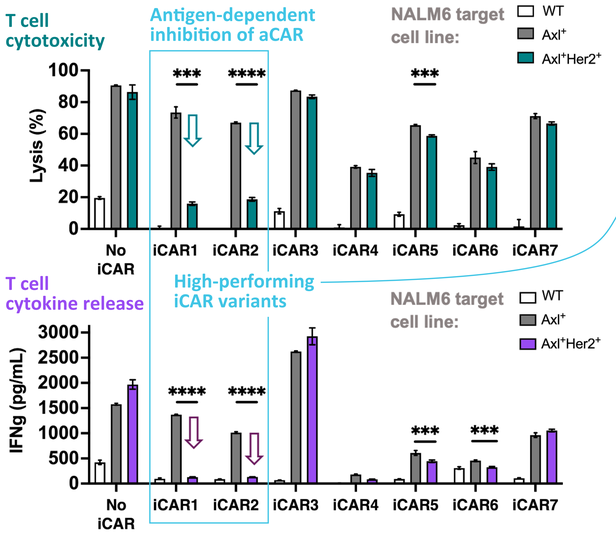
ICD 3.1
ICD 3.2
ICD 3.3
ICD 3.4
ICD 3.5

NOT gate prototype further validation

We have identified multiple iCARs that significantly reduce aCAR activation

NOT GATE in T cells with Axl aCAR and Her2 iCAR

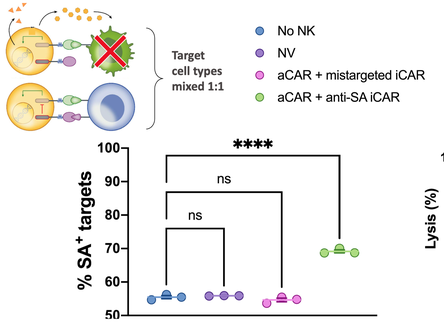
iCARs with different ICDs were screened in primary human CD3+ T cells. ICDs were built into anti-Her2 iCARs and co-transduced with anti-Axl aCARs. Here, Axl is a model tumor-associated antigen and Her2 is a model Safety Antigen. (Top) killing of NALM6 targets engineered to express different antigen combinations. For T cells without an iCAR on the far left, the anti-Axl aCAR kill Axl+ target cells to the same extent as Axl+ Her2+ target cells. On the other hand, T cells co-expressing aCARs with functional iCARs should kill Axl+ target cells but spare Axl+Her2+ target cells. In this screen, different iCARs had different levels of efficacy, with two high performers boxed in blue showing a large drop in killing. (Bottom) IFNg release further verifies the efficacy of these iCARs in suppressing activation in an antigen-dependent manner.



NOT GATE function is robust to complex target cell conditions

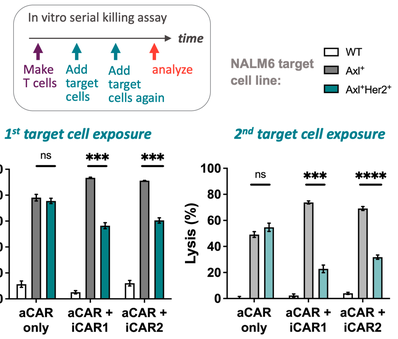
Mixed target cells +/- Safety Antigen

In patients with AML, tumor cells and healthy cells may be mixed in the same compartments, forcing NOT GATE gene circuits to quickly discriminate between them. We performed a mixed target assay in which NOT GATE CAR-NK cells were presented with a mixture of SEM cells that all expressed the tumor-associated antigen, but only 50% of them expressed the safety antigen. NOT GATE CAR-NK cells skewed the target cell population significantly towards those expressing the Safety Antigen, demonstrating that the gene circuit remains functional in mixed target cell conditions.

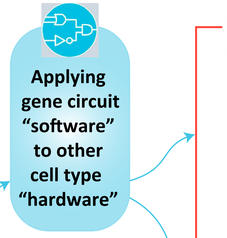


Repeated exposure to Safety Antigen

The NOT GATE must maintain suppressive capacity over multiple exposures to cells over time in a patient. If the iCAR loses function over time, it may result in aCAR toxicity. To simulate this scenario, we performed a serial killing assay with NOT GATE CAR-T cells. On the second exposure to target cells, the iCAR protected Safety Antigen-expressing cells at least as much as during the first exposure, demonstrating that the gene circuit continues to suppress aCAR toxicity after multiple target cell exposures.

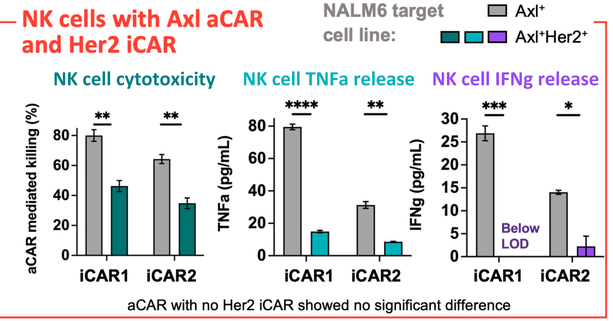


NOT GATEs can operate in multiple modalities and target antigen systems

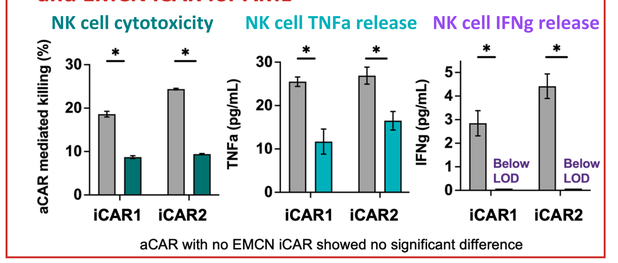


(Top) Transferring the same aHer2 iCAR and aAxl aCAR components that functioned in T cells into NK cells formed a functional NOT GATE that significantly reduced cytotoxicity and release of TNFa and IFNg in response to stimulation with the same NALM6 cell lines. (Bottom) Our gene circuits can be customized to potentially address many aspects of disease biology. We re-targeted the circuit for acute myeloid leukemia (AML) using endogenously expressed FLT3 as a tumor-associated antigen on SEM leukemia cells and EMCN as a Safety Antigen. The NOT GATE again significantly suppressed specific aCAR function in an antigen-dependent manner.

NK cells with Axl aCAR and Her2 iCAR



NK cells with FLT3 aCAR and EMCN iCAR for AML



Summary

Reduction of on-target/off-tissue toxicity

Our iCAR-based NOT GATE gene circuit can significantly reduces CAR-based NK cell killing by >50% in response to recognition of a Safety Antigen that signifies a healthy cell.

Multiple modalities and target antigen combinations

We have identified multiple iCARs that are effective in multiple therapeutic modalities (T cells, NK cells) and target antigen combinations (model systems, AML-specific antigens).

Robustness to complex conditions

NOT GATE function is robust to mixed target cell populations and repeated exposure to Safety Antigens on target cells, conditions which may be encountered *in vivo*.

Contact: nicholas.frankel@sentibio.com