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The Most Logical Approach to Improve CAR T Cell Therapy

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Combinational antigen recognition is the most logical way to improve the safety of cancer therapy. CAR T cells therapy, combined with synthetic biology, protein engineering, and bioinformatics, can perform advanced computations to enhance tumor targeting specificity.

Immunotherapy, including biologic and cellular approaches, is a critical area of focus for cancer therapeutics. Of the methodologies used to "upgrade" a patient's immune cells, one of the most promising has been chimeric antigen receptor (CAR) T cell therapy, wherein T cells are engineered to express synthetic antigen-specific receptors. A CAR consists of a ligand-binding domain fused to signaling domains from T cell receptor and costimulatory receptors. Antigen binding by the CAR triggers the signaling domain, leading to T cell activation and cell killing. Several CAR-T therapies have been approved by the FDA to treat different types of blood cancers such as acute lymphoblastic leukemia, large B cell lymphoma, and mantle cell lymphoma (Yip and Webster, 2018). However, finding one or more cell-surface biomarkers that uniquely specify cancer cells—distinguishing them unequivocally from healthy cells—has restricted the applicability of these cell-based therapeutics. Given that much of the current focus in cellular immunotherapy is on enhancing their activity, the simultaneous improvement in specificity is urgently needed to expand their therapeutic window and reduce toxicity. Addressing the targeting specificity problem is the focus of recent work published in *Cell Systems* (Dannenfelser et al., 2020) and *Science* (Lajoie et al., 2020).

The solution to the issue of cell targeting specificity is obvious yet elusive. It is a common knowledge that most human cell types, including cancer cells, are best characterized by a *combination* of features, rather than just one "magic bullet" biomarker. Therefore, an agent that can sense and logically respond to multiple features of the cancer is the most sensible approach to develop an efficacious and safe therapy. While obvious, most existing cancer therapeutic modalities, such as small molecules and biologics, are too simplistic to carry out logic computations. Therefore, given the computation limitation in most cancer therapeutics, the only choice is to continue identifying and targeting a unique feature of cancer cells, even though we know full well that the biology is working against this approach.

The concurrent advancement in cellular immunotherapy and synthetic biology, however, has drastically changed how we can target cancer cells. We are no longer constrained to target only one biomarker. Using multiple synthetic receptors, we and other have developed CAR systems or synthetic Notch receptor (synNotch) that fully activate the T cell only when two different antigens are presents on the cancer cells (AND gate) (Cho et al., 2018; Kloss et al., 2013; Roybal et al., 2016). Also, an inhibitory CAR (iCAR) using the intracellular domain of the PD-1 receptor has been developed (Fedorov et al., 2013). The iCAR, when combined with a conventional CAR, can form a 2-Input A AND NOT B gate. Inhibitory CARs could have a paradigm-shifting impact in cancer drug discovery because they would enable the use of "missing proteins" as a target, which is not possible by conventional therapeutics (one cannot kill cancer cells with small molecule drugs if the cancer cell is missing the target). However, these systems either require careful tuning of different receptor signaling strengths or lose the discriminatory capacity if closely related healthy cells are in close proximity (Srivastava et al., 2019).

Lajoie, Boyken, and Salter et al. (Lajoie et al., 2020) demonstrates an alternative AND gate and NOT gate CAR designs. Instead of using multiple receptors that integrate the signal intracellularly downstream of the receptor activation, Lajoie et al.'s system involves one receptor and performs the logic operation extracellularly through computationally designed protein logic circuits (Figure 1A). Using their previously designed Latching Orthogonal Cage–Key pRotein (LOCKR) switches (Langan et al., 2019), they improved the system to be colocalization dependent (Co-LOCKR). The resulting co-LOCKR AND gate circuit consists of a "cage" and "key" protein, each attached to an antigen-binding domain. The cage protein also contains a peptide that can bind to CAR T cells. The peptide, however, is sequestered by a latch domain on the cage protein. The key protein binds to the cage protein, causing a conformation change and exposing the peptide for binding to and activating the CAR. The cage and key proteins are designed to not interact in solution. However, colocalized to the cell surface by antigen-binding domains favors cage-key complex formation. They also demonstrated A AND NOT B CAR by adopting a decoy design. The decoy binds to the key protein, thus preventing it from activating the cage.

The Co-LOCKR design displays a remarkable logic computation capability with human immune cells. It also seems to have low off-target killing and robust activation *in vitro*. However, improvement is needed to translate this groundbreaking technology, especially for the NOT logic circuit. The overall T cell activity for the NOT logic CAR seems to be much

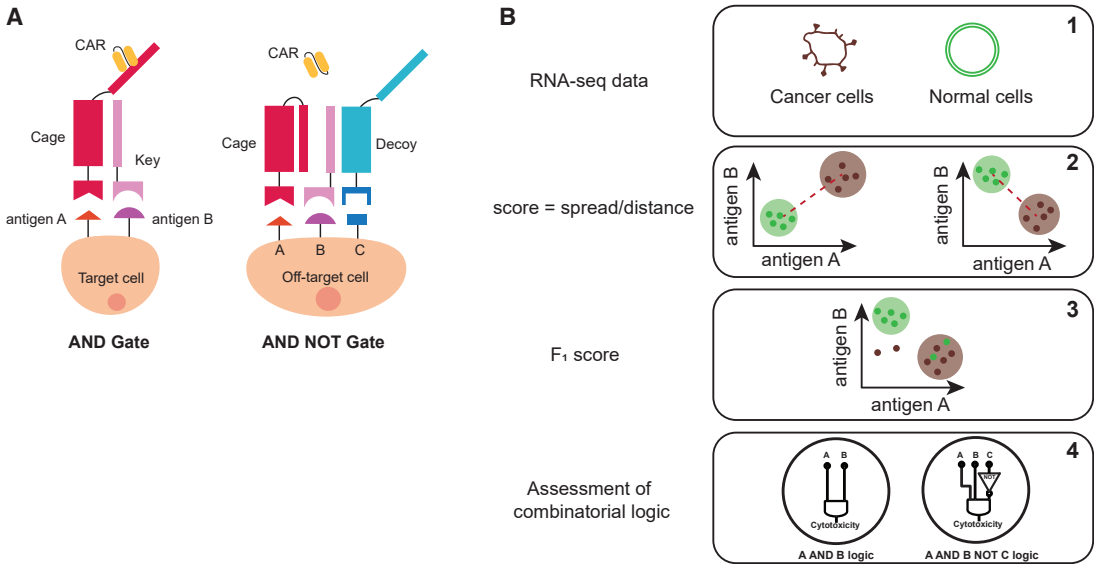

Figure 1. Co-LOCKR CAR T Cell Design and the Computation Framework for Determining the Discriminatory Power of Combinational Antigen Recognition

(A) Schematic of the co-LOCKR circuit design for AND and NOT gates.

(B) Work flow for evaluating the discriminatory recognition power of combinatorial antigen using RNA-seq data from cancer and healthy tissues.

lower than conventional CAR. Thus it is difficult to predict the effectiveness of the NOT gate CAR *in vivo*. Furthermore, both the decoy protein and its target may need to be in excess to efficiently out-compete the key from binding to the cage. Nonetheless, we anticipate that the Co-LOCKR framework can improve the combinatorial CAR T therapies by further assessing pharmacokinetics in physiological conditions and immunogenicity.

With synthetic receptors that can perform logic computation, the next issue to address is determining the right combination of target antigens. Previously, Perna et al. integrated proteomic and transcriptomic data to determine the best combination of antigens for CAR T cell therapy against acute myeloid leukemia (AML) (Perna et al., 2017). Dannenfelser et al. expanded the approach to many different cancers. They imported the predicted surface genes of both tumor cells and normal tissues from RNA-seq databases with batch correction (Figure 1B). They used the Davies-Bouldin metric for a clustering-based score to measure the ratio of "within cancer/tumor antigen expression divergence" to "cancer versus tumor separation." Proposing a score to capture off-target toxicity (precision) and inclusive on-tumor cytotoxicity (recall) when using a Boolean logic gate, they introduced a decision-tree model and presented how a combination of clinical and novel target antigens can exceed the current single target antigen approach in clinical trials. They validated their identified set of antigens for AND gate synNotch/CAR system developed by Lim group (Roybal et al., 2016) and were able to selectively kill the tumor expressing both antigens, but not when the tumor is missing one of the antigens. However, as acknowledged by the authors, their approach's primary limitation is the limited protein expression data and incomplete normal tissue expression dataset. The availability of these data in the future will undoubtedly improve their predictions.

Cancer has proven to be a formidable foe that is extraordinarily complex and remarkably relentless. We need therapeutic agents that can match the sophistication of cancer cells. CAR T cell developments, including the work by Lajoie et al. and Dannenfeser et al., are beginning to impart the intelligence and persistence into therapeutic agents required to level the playing field against cancer. While the synthetic receptor circuit designs may seem complicated, they are developed in direct response to the complex biology of many cancers. Therefore, one of our tasks ahead is to continue to invest in solutions that would turn innovative ideas outlined here into robust and effective therapies. The only way is through.

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DECLARATION OF INTERESTS

Patent applications have been filed on CAR designs (S.L, and W.W.W.). W.W.W. is a scientific co-founder, scientific advisory board member and shareholder of Senti Biosciences, and has received research support from Senti Biosciences.

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