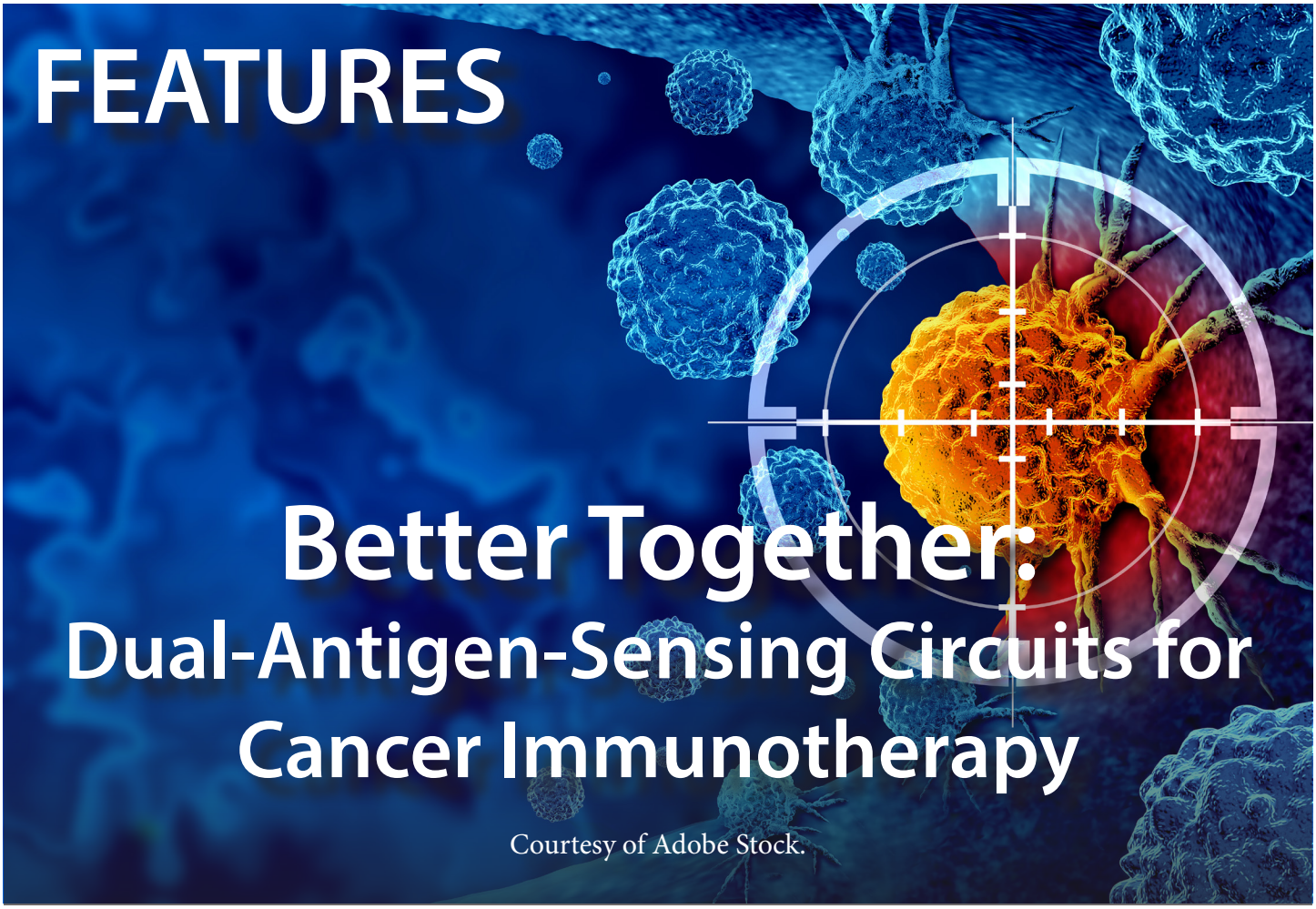

Better Together: Dual-Antigen-Sensing Circuits for Cancer Immunotherapy

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(figures courtesy of Lale Baylar ’28)

Introduction

Cancer is the second leading cause of death worldwide (Weiss, 2021). To address this global challenge, researchers have developed a toolkit of varied treatment options including chemotherapy, which uses drugs to kill cancer cells; radiation therapy, which directs high-energy rays at tumors; hormone therapy, which inhibits tumor growth; and surgery, which removes tumors directly. One particularly promising and versatile approach to treatment, immunotherapy, harnesses the patient’s own immune system to fight cancer. Cellular immunotherapy—also known as adoptive immunotherapy or T lymphocyte (T cell) transfer therapy—involves engineering the patient’s immune cells to make them better at recognizing and attacking tumors. Cellular immunotherapy is a “living drug” that can potentially remain in the body for years, continuing its anti-tumor activity if relapse occurs (Kumar et al., 2021).

Cellular immunotherapy relies on cytotoxic T cells, a class of white blood cells that kill foreign, infected, and cancerous cells in the body (Richard et al., 2023). T cells identify targets using specialized receptors that recognize specific proteins—called antigens—found on threatening cells (Waldman et al., 2020). Antigen recognition triggers complex networks of signaling proteins and transcription factors within the T cell that mediate its gene expression, thereby activating the T cell and enabling it to kill the antigen-presenting cell.

The immune system naturally kills most spontaneous cancer cells, keeping them from growing into tumors. But cancer is elusive, and the selective pressure exerted by the immune system can cause cancer cells to adapt to present less and less of the nonessential antigens that T cells use as targets, a process called antigen loss (Mishra et al., 2024). If the target antigen has been lost, tumors can escape detection by the immune system. Cellular immunotherapy attempts to address tumor escape by engineering T cells to recognize custom antigens of the designer’s choosing—for instance, essential proteins that

tumors are less likely to repress—thereby reducing reliance on default nonessential antigen targets. Scientists equip patients' own T cells with genes encoding custom-designed synthetic receptor proteins that recognize a particular tumor antigen target. These engineered receptors direct the T cells to attack cancer.

Successes and Challenges

Antigen Specificity

A major advantage of cellular immunotherapy over chemotherapy and radiation therapy is that it targets tumors with cellular-level precision, minimizing off-target damage to healthy cells. T cells' engineered receptors require antigen detection to activate cell-killing responses, so non-cancerous tissue is only harmed if the chosen tumor antigen target also appears on healthy cells—a situation called "on-target, off-tumor" toxicity (Flugel et al., 2023). Still, some safe-to-use immunotherapies involve manageable amounts of on-target, off-tumor toxicity, such as treatments for B cell cancers that target both malignant and healthy B cells.

Tumor-specific antigen targets (i.e., antigens found only on cancer cells) are desirable because targeting them poses no risk of on-target, off-tumor toxicity. Ideally, targets should be exclusively and uniformly expressed by cancer cells. Without exclusivity, T cells could attack healthy cells that coincidentally presented the antigen; without uniformity, a subset of cancer cells that lacked the antigen could escape undetected. However, discovering true tumor-specific antigens is difficult, so some immunotherapies instead target tumor-associated antigens expressed at high levels in cancer cells and lower levels in healthy cells (Okarvi & AlJammaz, 2019).

Furthermore, antigen loss remains a challenge even though the antigens that cellular immunotherapy targets are often harder to lose than the antigens that T cells naturally target. As long as an antigen is nonessential, tumor cells may downregulate or remove it (Mishra et al., 2024).

Solid Tumors

Cellular immunotherapy has successfully been used to treat liquid cancers, but it demonstrates decreased efficacy against solid tumors such as sarcomas, carcinomas, and glioblastomas (Dagar et al., 2023; Guzman et al., 2023). Solid tumors are particularly difficult to treat because they are surrounded by a network of extracellular molecules and non-cancerous tissue that supports tumors, collectively termed the tumor microenvironment (Tormoen et al., 2018). This tumor microenvironment hinders T cells by suppressing their activity, depriving them of nutrients, and physically limiting their access to tumors. Researchers are actively developing and testing new strategies to overcome the challenges posed by the microenvironment.

Introducing Two Synthetic Receptors: SynNotch and CAR

The ongoing challenges of antigen specificity and the solid tumor microenvironment necessitate novel approaches in cellular immunotherapy. A recent innovation combines the activity of two particularly promising receptor proteins—the chimeric antigen receptor (CAR) and the synthetic Notch (synNotch) receptor—in order to more precisely target tumors. Both of these proteins can recognize tumor antigen targets and activate therapeutic responses in T cells, but each has a distinct function when combined.

Chimeric Antigen Receptors

One of the most well-researched synthetic receptor proteins used in cellular immunotherapy is the CAR. The first approved human "gene therapy" in the United States was a type of CAR T cell therapy, Kymriah, that was approved by the FDA in 2017 to treat acute lymphoblastic leukemia (Awasthi et al., 2023). CARs enable the activation of T cells' built-in cell-killing mechanisms in response to user-defined antigen targets (**Fig. 1**). Lodged in the cell membrane, CARs are proteins that comprise (1) an extracellular receptor region that recognizes the tumor antigen target; (2) a transmembrane region that anchors the protein and relays signals from the receptor to the inside of the cell; and (3) intracellular signaling regions that induce T cell activation (Irvine et al., 2022).

Synthetic Notch Receptors

SynNotch receptors, developed more recently than CARs, are a new generation of engineered synthetic

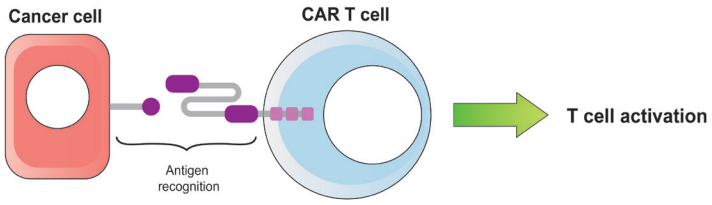

Figure 1. An antigen-presenting cancer cell binds to a CAR T cell, triggering T cell activation.

receptor proteins with exciting implications for the future of immunotherapy. Like CARs, synNotch receptors are transmembrane proteins that recognize user-defined antigen targets and trigger cellular responses. But unlike CARs, antigen recognition by a synNotch receptor activates the expression of customizable, potentially non-native genes. When the antigen target binds to the extracellular region of the synNotch receptor, the intracellular region releases a transcriptional regulator into the cytoplasm that traffics to the nucleus and activates DNA transcription (**Fig. 2**) (Roybal et al., 2016b).

What makes synNotch receptor proteins so promising is their ability to trigger the expression of customizable genetic response programs that are not naturally found in T cells. Researchers choose which genes the receptor

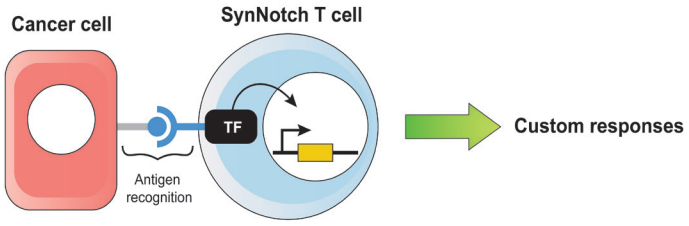

Figure 2. An antigen-presenting cancer cell binds to a synNotch T cell, triggering custom therapeutic responses (adapted from Roybal et al., 2016b).

controls by inserting chosen DNA into the T cell's nucleus to be transcribed upon receptor activation. These genetic programs have the potential to perform a wide variety of functions, such as increasing T cell toxicity against tumors (Bonamino et al., 2022; Golubovskaya & Wu, 2021), inducing T cell proliferation (Li et al., 2022), and helping T cells navigate the solid tumor microenvironment (Foeng et al., 2022).

Combining Receptors in Dual-Antigen-Sensing Circuits

CARs and synNotch receptors each play important roles in the toolkit of cellular immunotherapy, recognizing tumor antigens in order to either activate T cells or trigger the expression of custom genetic programs with diverse functions. However potent these proteins are alone, recent studies have shown that they may be even more effective when combined.

Two of the most significant hurdles in CAR T cell therapy for the treatment of solid tumors are (1) ensuring that only cancer cells are targeted and (2) avoiding antigen loss and tumor escape (Choe et al., 2021). In an attempt to overcome these challenges, Dr. Wendell Lim's laboratory at the University of California, San Francisco engineered T cells that express both synNotch receptors and CARs in order to recognize two different tumor antigen targets. Together, synNotch receptors and CARs form a molecular AND-gate circuit that activates T cells only when both antigens are present (Roybal et al., 2016a).

In dual-antigen-sensing T cells (**Fig. 3**), the synNotch receptor has been engineered to recognize a particular antigen: antigen A. If antigen A is present on a nearby cancer cell, the antigen binds to the synNotch receptor, activating the expression of genes encoding a CAR. This CAR has been engineered to sense a different antigen: antigen B. If the cancer cell that presented antigen A also presents antigen B, then the newly expressed CAR will recognize antigen B and induce T cell activation to kill the cancer cell (Roybal et al., 2016a). SynNotch-CAR circuits broaden the range of targets for cellular immunotherapy by opening the doors to antigens that may not be tumor-specific on their own but that become specific when combined with a second target (Choe et al., 2021).

In vivo mouse studies have demonstrated that synNotch-CAR T cells spare non-cancerous cells that express a single antigen (i.e., only antigen A or only antigen B) while successfully killing tumors that express both antigens at the same time (Roybal et al., 2016a). Additional studies found that synNotch-CAR T cells were better at controlling

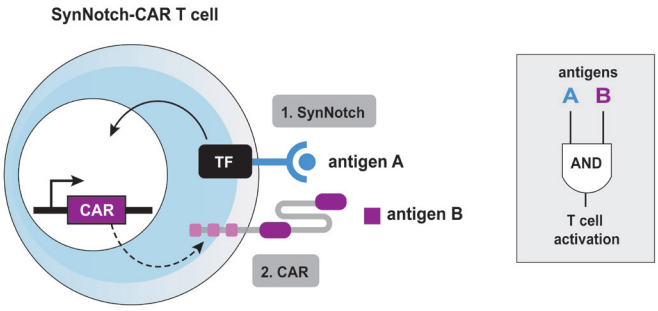

Figure 3. SynNotch-CAR T cells recognize two antigen targets in order to induce T cell activation (adapted from Roybal et al., 2016a).

solid tumors in mice than traditional CAR-only T cells and that the synNotch-CAR T cells did not damage healthy tissue (Hyrenius-Wittsten et al., 2021; Choe et al., 2021).

Conclusion

Uniting CARs and synNotch receptors to create T cells that require two antigen targets for activation is a powerful new strategy already being used to treat solid tumors in mice. SynNotch-CAR circuits help overcome

the challenge of antigen specificity by enabling precise AND-gate antigen recognition, allowing researchers to choose from a wider variety of antigen targets that may reduce the risk of on-target, off-tumor toxicity. Since the particular antigens that synNotch receptors and CARs target are up to the engineer, this technology could potentially be applied to many different types of cancer. Although further research, clinical testing, and approval are still necessary before this technology can be used to treat disease in humans, synNotch-CAR circuits are a promising innovation in the ongoing fight against hard-to-treat solid cancers. This genetic engineering-based technology may further enhance cellular immunotherapies when combined with other approaches such as protein, chemical, biological, or materials engineering (Irvine et al., 2022).

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