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Review

# Recent progress of gene circuit designs in immune cell therapies

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## SUMMARY

The success of chimeric antigen receptor (CAR) T cell therapy against hematological cancers has convincingly demonstrated the potential of using genetically engineered cells as therapeutic agents. Although much progress has been achieved in cell therapy, more beneficial capabilities have yet to be fully explored. One of the unique advantages afforded by cell therapies is the possibility to implement genetic control circuits, which enables diverse signal sensing and logical processing for optimal response in the complex tumor microenvironment. In this perspective, we will first outline design considerations for cell therapy control circuits that address clinical demands. We will compare and contrast key design features in some of the latest control circuits developments and conclude by discussing potential future directions.

## INTRODUCTION

Cells are sophisticated information processing systems that can sense diverse environmental signals, perform complex computations, and produce a wide array of outputs, such as gene expression, signaling molecule secretion, morphological changes, and cell growth (Lim, 2010). Furthermore, a number of cell types have evolved specialized capabilities to survive in different environments and perform various tasks. These features establish cells as excellent candidates for smart therapeutics with enhanced safety and efficacy. Indeed, several cell types have been evaluated for the development of cell therapies, including bacteria and stem cell therapies. In particular, one of the most important classes of cell for therapeutics development is the human immune cell (Bailey and Maus, 2019). For instance, T cells genetically engineered with a chimeric antigen receptor (CAR) have demonstrated potent anti-cancer cytotoxicity in the clinics, leading to five Food and Drug Administration (FDA)-approved therapies for B cell malignancies (FDA, U.S., 2021a, 2021b, 2017a, 2020, 2017b).

Although promising, many challenges need to be addressed before we can realize the full potential of immune cell therapies. One of the most pressing concerns for cellular immunotherapy is toxicity caused by the overactivation and off-tumor targeting of the engineered immune cells. Moreover, the heterogeneity and constant evolution of many diseases demands a dynamic intervention rather than a static, one-time treatment (Marusyk et al., 2012; Meacham and Morrison, 2013). Furthermore, the efficacy of immune cell therapies needs further improvement, as exemplified by the challenges CAR T cells face against solid tumors. Advanced therapeutic cell designs with enhanced precision and control are necessary to address these issues. Most importantly, the challenges in safety and efficacy need to be solved simultaneously to create effective treatments.

## CELL-AUTONOMOUS VERSUS EXOGENOUS CONTROL: DESIGN CONSIDERATIONS

Unlike most therapeutic modalities, cell therapies can be equipped with sophisticated gene circuits to improve their targeting specificity, safety, and efficacy. Although there are many different types of gene circuits, they can be broadly classified into two classes: cell-autonomous and exogenous control (Figure 1). Cell-autonomous control gene circuits rely on signals from within the engineered immune cells or the native environment. In contrast, exogenous control gene circuits rely on signals from external reagents, such as small molecules, lights, or ultrasound. These circuits are not mutually exclusive and can be deployed together.

When deciding to employ gene circuits to improve immune cell therapies, it is important to consider the relative strengths and weaknesses of each class of gene circuits. Cell-autonomous circuits are attractive because they can operate without user intervention. This feature may be necessary because some features are not amenable to manual control, such as precisely locating a tumor based on a combination of molecular markers. However, as we have witnessed from autonomous vehicle development, a completely self-operating system may present challenges that require monitoring and adaptation. In clinical settings, unpredictability is far from acceptable. As such, the ability to apply exogenous control to engineer cell therapies will be highly desired.

One of the key considerations for exogenous control circuits is the choice of the input control. The input could be delivered systemically, such as a small molecule, or applied in a highly localized manner, such as light or ultrasound. Small molecules are easy to administer but may have toxicity or poor pharmacokinetic properties. In contrast, light and ultrasound provide non-invasive and precise spatiotemporal control. Still, continuous

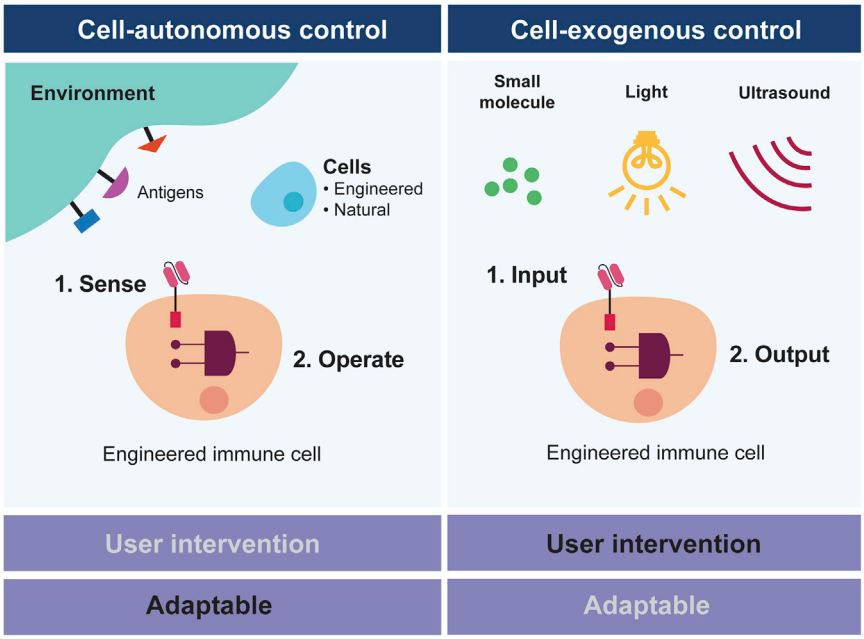


**Figure 1. Comparison between cell-autonomous and exogenous control**

Autonomous control of engineered immune cells can sense and respond to the environment or internal signals, whereas exogenous control enables user intervention of the engineered cells through various types of inputs.

delivery of light and ultrasound to the patient, which could be required to ensure sustained immune cell function, may not be practical.

A previous review by Lim and June from 2017 has highlighted the pioneering work in this space (Lim and June, 2017). Here, we would like to bring forth some of the latest developments in genetic circuits for immune cell therapy. We will emphasize discussing the pros and cons of these gene circuits. Finally, we will provide an outlook on how gene circuits can lead to the next generation of smart cell therapies.

## CELL-AUTONOMOUS CIRCUIT FOR THERAPEUTIC IMMUNE CELLS

Cell-autonomous gene circuits can sense and respond to input signals within the patient. There are several types of input signals that gene circuits have been designed to sense: the combination of antigens from target and healthy cells, intracellular cell states, and tumor microenvironment. These circuits provide logic and feedback control for more precise temporal and contextual responses of the engineered immune cells.

### Receptor logic circuits

Combinatorial antigen recognition is the most logical approach to improve tumor targeting and reduce the potential toxicity of cancer cell therapies, as often no single antigen exists to uniquely classify cancer cells. Among various receptor logic circuits applied to immune cells (Ruella et al., 2016; Grada et al., 2013; Hegde et al., 2016; Zah et al., 2016; Lanitis et al., 2013; Kloss et al., 2013; Fedorov et al., 2013), we will highlight three of the most advanced logic circuits—split, universal, programmable CARs (SUPRA CARs), synthetic Notch (synNotch), and Colocalization-dependent Latching Orthogonal Cage/Key pRoteins (Co-LOCKR)—that have been applied to perform up to 3-input AND, NOT, and OR logic (Roybal et al., 2016a; Cho et al., 2018, 2021; Zhu et al., 2022; Lajoie et al., 2020).

### CAR circuits

A traditional CAR is composed of an antigen-binding domain fused to key intracellular signaling domains from the T cell receptor (TCR) (e.g., CD3ζ or CD3ε) and costimulatory receptors (e.g., CD28 or 4-1BB). Signaling from the TCR and costimulatory receptor domains are needed for full T cell response. Similarly, for inhibitory CAR (iCAR), intracellular signaling domains from inhibitory receptors have been employed to inhibit the signal from the traditional activating CAR (aCAR) (Fedorov et al., 2013; Richards et al., 2021; Tao et al., 2020; Hamburger et al., 2020; Hwang et al., 2021; Sandberg et al., 2022).

The core design principle of a multi-input CAR logic circuit is to create separate CARs to perform the functions of TCR, costimulatory, and inhibitory receptors separately with different antigen targets (Figure 2A). In essence, a unique CAR is created for each signaling pathway. The signal integration will occur intracellularly through the endogenous signaling network. Although conceptually simple, the challenge in implementing the CAR logic circuit is to ensure the signaling strength from each receptor is properly calibrated. For instance, if the aCAR signaling is too strong, the iCAR may not be able to inhibit the signal.

One of the most direct ways to modulate the CAR signaling strength is to control the number of receptors present on the cell surface. A split universal CAR configuration is the most convenient approach to modulate the number of functional receptors on the cell. A split CAR design is composed of a universal receptor and an adaptor protein that binds both the universal receptor and the target cell. By varying the concentration of the adaptor protein, one can modulate the number of functional CARs and therefore the strength of the signaling.

Many split CAR designs have emerged within the past few years (Urbanska et al., 2012; Lohmueller et al., 2017; Ma et al., 2016). However, the most versatile system is the SUPRA CAR system, which offers the most diverse, orthogonal set of leucine-zipper universal CAR receptors (zipCARs), and leucine-zipper "adaptor" domains that bridge the zipCAR receptors to a variety of antigens specified by an single chained variable fragment (scFv) domain (zipFv) (Figure 2B). The SUPRA CAR system showed tunable CAR activation with zipFv titration and antigen-specific activation. Taking advantage of leucine-zipper pairing orthogonality, a variety of logic operations (OR, AND, NOT) with zipFvs of varying affinity

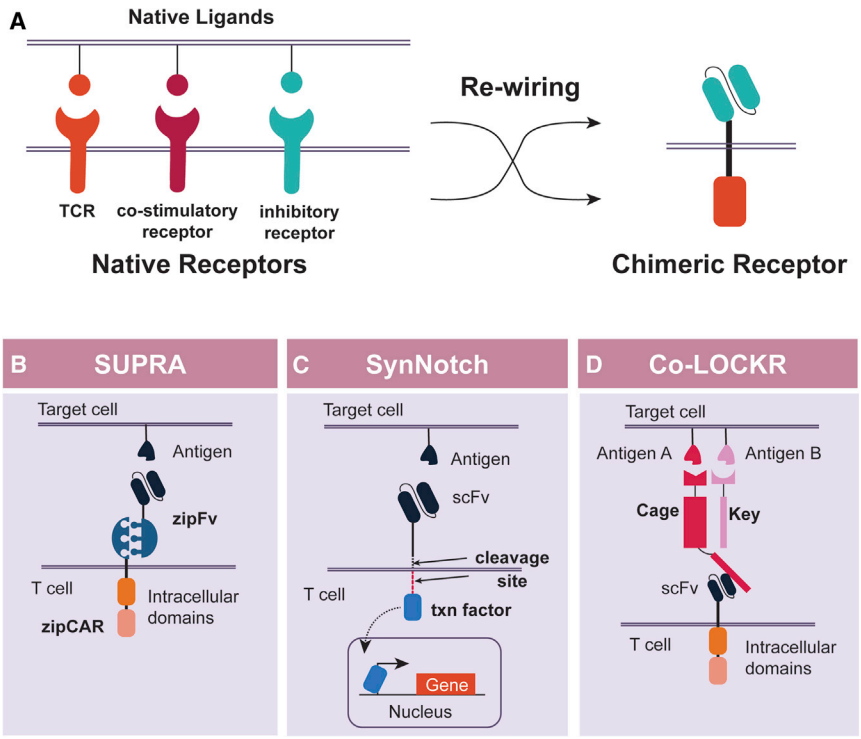


**Figure 2. Schematics of chimeric antigen receptors and receptor logic circuits**

(A) Chimeric antigen receptors are derived from native receptors, exchanging their intracellular domains and extracellular domains to rewire their targeting specificity.

(B) SUPRA CAR consists of zipFv and zipCAR. Swapping the zipFv allows the targeting of various antigens by the same zipCAR.

(C) SynNotch receptor can induce gene expression in response to the desired antigen. Once the antigen is bound to the scFv domain, membrane-bound transcription factor (txn factor) will be released to induce the gene expression.

(D) Co-LOCKR system consists of a CAR and two adaptor proteins: Cage and Key. Only when cage and key are bound on the same target cell can the cage domain be exposed to activate the CAR.

against multiple antigens was demonstrated *in vitro* and *in vivo* (Cho et al., 2021).

Although SUPRA CAR has high modularity, it is a more complicated therapy that consists of protein and cell therapy. Because the adaptor molecule is a protein, zipFv may have less permeability into the desired tissue, a shorter half-life, and potential unknown immune responses. The appropriate indication of the SUPRA CAR will likely be context dependent.

## SynNotch

The synNotch receptor developed by the Lim group represents a distinctive approach to achieve logic in CAR T cells (Morsut et al., 2016; Roybal et al., 2016b). A synNotch receptor is composed of an extracellular antigen-binding domain, followed by a proteolytic transmembrane core from the Notch receptor, and a programmable transcription factor against the target gene promoter. Ligand binding to the synNotch receptor leads to the cleavage of the core Notch domain and release of the transcription factor and transcription activation (Figure 2C). The synNotch receptor is a programmable surface ligand inducible gene expression system. The Lim lab has previously employed synNotch to reprogram immune cells and design complex tissue patterns (Toda et al., 2018). Recently, a collection of modular and humanized proteolytic-based receptors similar to the synNotch has been developed by Roybal and colleagues (Zhu et al., 2022). Using mainly human components will minimize immunogenicity and facilitate their clinical translation.

The synNotch-based logic circuit employs an "IF-THEN" logic for which the activation of the synNotch leads to the expression of a CAR or an apoptotic gene to achieve AND or NOT logic, respectively (Williams et al., 2020; Roybal et al., 2016b). The synNotch and the CAR can each target different antigens, leading to multi-input logic circuits. The AND logic performance resulting from the synNotch-based circuit seems to enable improved specificity, even against glioblastoma, a solid tumor that is infamous for its high antigen heterogeneity (Choe et al., 2021).

However, a synNotch-based circuit does not require the antigens to be present on the same cell. Once the CAR is expressed, the antigen for the synNotch is no longer necessary. Therefore, if the off-target healthy cells expressing the antigen for the CAR are near the intended tumor cells, they could also be eradicated (Srivastava et al., 2019).

## Co-LOCKR

The Co-LOCKR CAR system is also based on a split CAR design. However, the Co-LOCKR system only uses one receptor (Figure 2D). The logic operation is achieved through a set of computationally designed adaptor proteins that can interact with each other and modulate how the adaptor proteins bind to the CAR in the presence of target antigens. The core of the Co-LOCKR system is the "cage" and "key" proteins, each with an antigen-binding domain. The cage protein also contains a peptide that can bind and activate the CAR T cells. The peptide domain of the cage, however, is sequestered by a latch domain. When the key protein binds to the cage protein, it causes a conformational change and exposes the peptide for binding, which allows for activation of the CAR. The cage and key proteins are designed to not interact in solution. Instead, the equilibrium favors cage-key complex formation once they are colocalized to the cell surface by antigen-binding domains. Co-LOCKR switches have been utilized in CAR designs to target up to three different antigens on cancer cells. This split CAR system can also function with AND, OR, and even advanced logic such as A AND B NOT C (Lajoie et al., 2020). The Co-LOCKR design does not require the balancing of intracellular signaling domains, but rather requires the presence of the "key" protein to open up the "cage." However, the employment of a decoy "key" protein to generate NOT logic has a limitation in that the logic will be dependent on the decoy protein abundance.

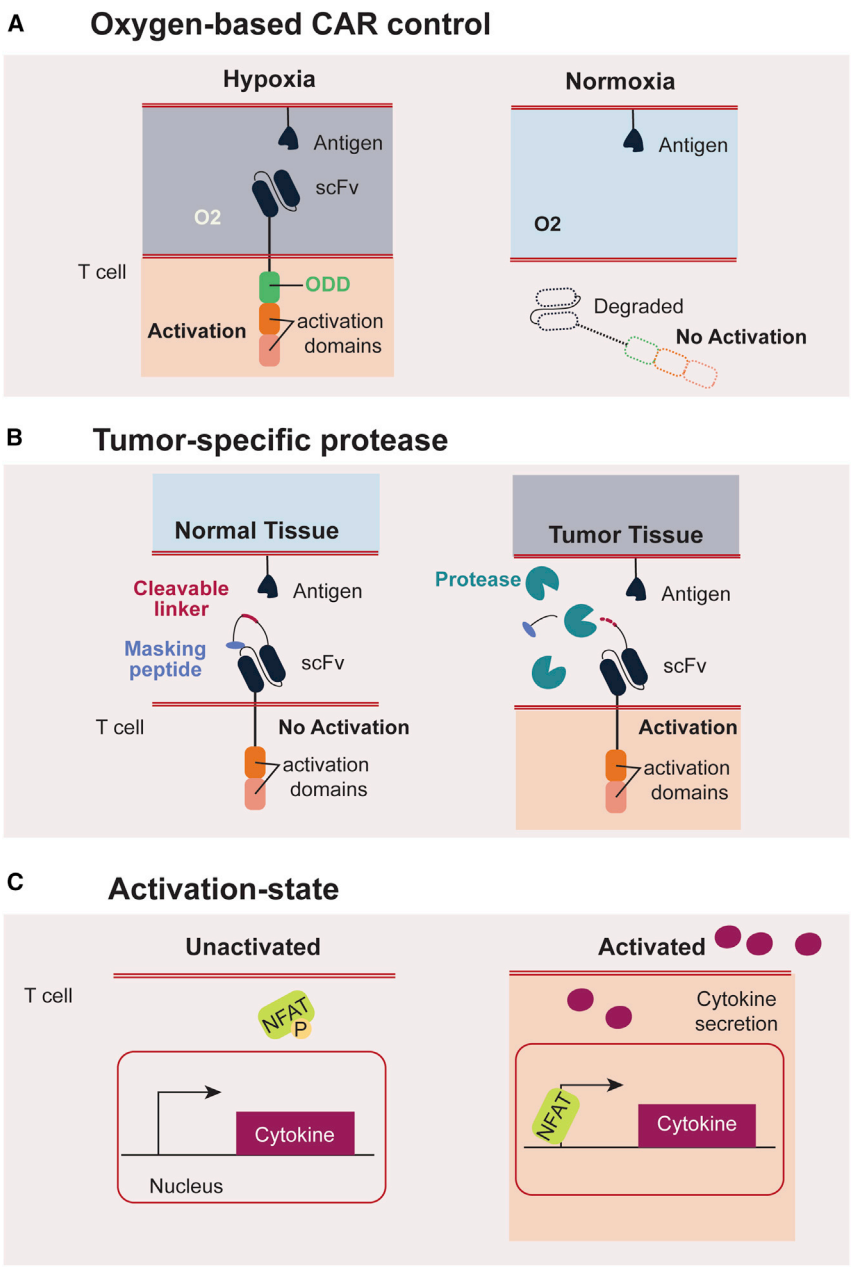


**Figure 3. System designs for cell-state-based control**

(A) CAR with an oxygen-dependent degradation (ODD) domain, for which its stability is dependent on hypoxia. The ODD will be degraded under normal oxygen concentration, resulting in the degradation of the CAR.

(B) A CAR design that can be activated only under the presence of tumor-specific protease. The scFv is masked by a cleavable linker and a masking peptide, and the protease can cleave off the linker to expose the scFv so that the CAR can be activated against the target antigen.

(C) Activation status of the immune cell can be utilized to control further cytokine generation. Once the T cell is activated, the NFAT is dephosphorylated and translocated into the nuclease to induce the target cytokine transcription.

## Cell-state-based control

Cell surface antigens are not the only signals capable of redirecting immune cell cytotoxicity. The tumor microenvironment is often immunosuppressive, as it is concentrated with immune inhibitory factors and metabolites to limit cytotoxic immune function (Tang et al., 2021; Zou, 2005). Immune cells can be engineered to detect some features of the tumor microenvironment and produce factors to augment anti-tumor activity, representing a powerful strategy to overcome the tumor microenvironment. Although the interventions based on cell states may enhance the specificity of the treatments, there is a possibility that they could also lead to weaker activity. As the tumor shrinks, the representative cell states may also be diminished, thus limiting the therapy's potency or specificity. Therefore, balancing activity and specificity would be crucial for cell-state-based control designs.

## Oxygen-based CAR control

A hallmark of solid tumors is hypoxia (low oxygen tension), often localized due to irregular vasculature and dense cell mass (Chang and Lai, 2020). Therefore, hypoxia can serve as an input signal to further increase tumor-targeting specificity for CAR T cell therapy. One strategy to achieve a hypoxia-inducible CAR structure is to fuse an oxygen-dependent degradation (ODD) domain to a CAR, rendering the stability of the CAR dependent on hypoxia (Juillerat et al., 2017) (Figure 3A). This ODD-fused CAR demonstrated hypoxia-induced cancer cell killing *in vitro*, but substantial basal killing under normal oxygen levels was also observed. An alternative approach that builds upon the ODD-fused CAR concept uses a synthetic hypoxia-inducible promoter to control the ODD-CAR transcription, thus providing two levels of control in CAR activity. The HypoxiCAR T cell (Kosti et al., 2021) can infiltrate tumors, leading to partial tumor clearance without cytokine release syndrome (CRS), a known problem associated with some CARs such as anti-Her2 (Morgan et al., 2010). Further characterization of the HypoxiCAR performance under hypoxic conditions in normal cells for a prolonged period is needed to establish the safety control of this hypoxia-regulatable CAR T cell therapy.

## Tumor-specific protease

Tumors often secrete proteases to promote invasion and facilitate various stages of tumor development. As such, tumor-specific proteases can be a marker for cancer diagnostics and therapeutics development. Recently, Han et al. (Han et al., 2017) developed a masked anti-EGFR CAR T by adding a

masking peptide with a proteolytic site before the scFv domain (Figure 3B). The masking peptide blocks the antigen-binding site by default, thus preventing CAR activation. However, in the presence of a tumor-specific protease, the masking peptide is cleaved, thus exposing the scFv and allowing antigen binding and activation of the CAR T cells. The masked CAR T cells had reduced activity in the absence of proteases despite surrounding target antigens *in vitro*. Masked CAR T cells demonstrated activity similar to unmasked CAR T cells in a subcutaneous human lung cancer xenograft model, indicating the cleavage of the masking peptide. An analysis of the off-tumor activity of the masked CAR T in relevant animal studies will further support the safety in the clinic.

### Activation cell state

Immune modulatory factors such as cytokines are essential in maintaining immune homeostasis and combating tumors and infection. As such, the application of cytokines like IL-2 and IL-12 as anti-cancer therapies is under investigation. Furthermore, cytokine administration has been explored as a combination therapy to enhance CAR functionality (Bell and Gottschalk, 2021; Hoyos et al., 2010; Liu et al., 2019). However, systemic cytokine administration can cause serious side effects (Berraondo et al., 2019; Ahmadzadeh and Rosenberg, 2006; Refaeli et al., 1998; Gattinoni et al., 2005; Krenciute et al., 2017; Yang et al., 2012). Therefore, it would be desirable for the CAR T cells to produce the cytokines only in the tumor microenvironment to minimize systemic toxicity. One approach to ensure localized cytokine production is to make it conditional on CAR activation. The nuclear factor of activated T cells (NFATs)/IL-2 composite promoter, which has long been used as a reporter of T cell activation (Shapiro et al., 1998; Jain et al., 1995; Riegel et al., 1992), was employed to control cytokine production in CAR T cells (Figure 3C). IL-12, IL-18, and IL-21 have been explored so far (Koneru et al., 2015; Chmielewski and Abken, 2017; Zimmermann et al., 2020; Guo et al., 2022; Štach et al., 2020), establishing the CAR T cells as a cytokine factory.

## EXOGENOUS GENE CONTROL CIRCUITS FOR THERAPEUTIC IMMUNE CELLS

One of the most important goals of exogenous gene control circuits is to enhance the safety of the engineered immune cells by limiting T cell activity in the event of adverse side effects or to improve tumor-targeting specificity. Therefore, the pharmacokinetics and safety profile of the inducer are two of the essential parameters in designing the inducible system. From a clinical perspective, implementing a safe, clinically approved inducer has extensive benefits, facilitating the introduction of novel CARs with enhanced safety profiles in the market. In addition to safety, an added benefit of using an inducible switch is increased durability. Weber et al. showed that transiently stopping tonic receptor signaling through a drug-gated CAR can rescue T cells from exhaustion, thus improving their *in vivo* persistence and ultimately anti-tumor activity (Weber et al., 2021).

Currently, there are three classes of exogenous gene control circuits, categorized by the type of inducers: small molecules, light, and ultrasound. When implemented in immune cells, each system acts as an ON or OFF switch, with the exogenous inducers modulating this change in response. Most of these systems, with the exception of kill switch or recombinase-based systems, do not have memory. As such, the inducer needs to be present continuously to maintain the ON or OFF state. Therefore, the toxicity and delivery method of the inducer is important. Furthermore, the decision to create an ON or OFF switch is dictated by the property of the components used in the system. Whether an ON or OFF switch is more desirable clinically, however, remains unresolved. We posit that an ON switch, which requires constant induction, is best suited when the output that it controls may become toxic at a high level (e.g., a pleiotropic cytokine or an overactive CAR), thus requiring fine-tuning and careful regulation. In contrast, an OFF switch, which stays ON without any inducer, is best used with an output that is relatively safe (e.g., a well-behaved CAR) and only needs to be turned OFF in case of severe side effects. However, when the output is no longer needed, the ON switch has the advantage that it can be shut off simply by withdrawing the inducer.

### Small molecules

The simplest way to generate drug-gated CAR T cells is to control the activity of the CAR directly. Common mechanisms used to achieve drug-gated control rely on inducible assembly or stabilization of the receptor. The assembly mechanism typically involves splitting the CAR into antigen recognition and signaling domains. A small molecule is used to either assist (ON switch) or disrupt (OFF switch) the assembly of the components (Li et al., 2022; Jan et al., 2021; Wu et al., 2015; Labanieh et al., 2022). The stabilization mechanism involves fusing a small molecule controllable degradation domain (degron) to the CAR. These degrons can unfold or cleave the CAR, and the binding of the inducer can stabilize the degron or inhibit the proteolysis (ON switch; Figure 4A). Some degrons will recruit endogenous proteolysis machinery in the presence of the small molecule inducer (OFF switch; Figure 4B). Recently, inducible CAR systems based on non-structure 3 (NS3) protease from the hepatitis C virus (HCV) have been developed (Li et al., 2022; Labanieh et al., 2022; Israni et al., 2021). The advantage of the NS3 system is that it can be regulated by clinically approved protease inhibitors, which have a favorable safety profile. Usually, a given inducible system can only leverage either the assembly or the stabilization mechanism. However, some systems, such as the versatile protease regulatable CAR (VIPER CAR) or the lenalidomide system (Li et al., 2022; Jan et al., 2021; Labanieh et al., 2022), can leverage both mechanisms to create ON and OFF switches using the same inducer. Furthermore, Li et al. have shown that the NS3-based system can be combined with other CAR designs (e.g., SUPRA or the lenalidomide system) to create multiplexed control circuits that could improve the safety and specificity of the CAR T cell therapy.

An alternative and more flexible approach to creating regulatable immune cell therapies is to deploy drugs for tuning CAR or therapeutic gene expression. The most prominent of such systems is the Tet-on transcription system (Drent et al., 2018; Gu et al., 2018; Sakemura et al., 2016). In this system, CAR is transcribed only in the presence of doxycycline, though some leaky expression has been observed. Moreover, high levels of TetR proteins can be toxic due to off-target binding in the genome (Kramer and Staveley, 2003; Pfeiffer et al., 2010; Rezával et al.,

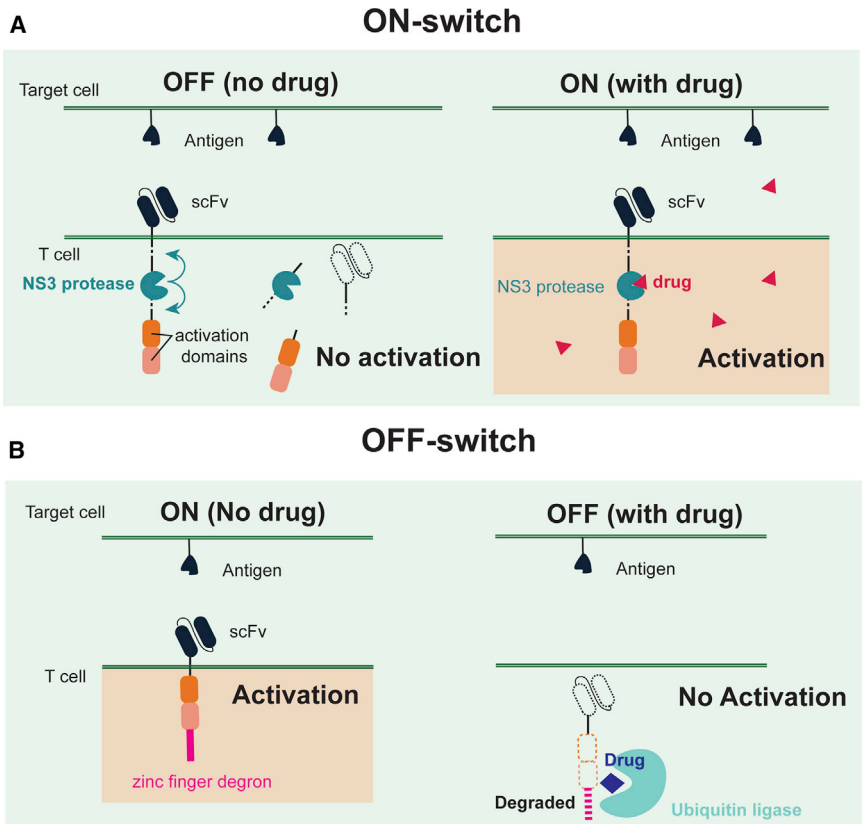


**Figure 4. Exogenous cell control with ON and OFF switches**

(A) Representative ON switch incorporating an NS3 protease to control the CAR activity. CAR will be stabilized only under the presence of a drug that can inhibit the protease activity to enable signal transduction.

(B) Representative OFF switch using the zinc-finger degron motif and a synthetic ubiquitin ligase. A drug that can induce the dimerization between the degron and the ligase will signal the CAR for degradation, so the T cells cannot be activated in the presence of the drug.

2007). Programmable synthetic transcription factors, such as those based on zinc finger or CRISPR, could provide a safer option to mitigate off-target effects. In particular, the synthetic zinc-finger transcription regulators (synZiFTRs) have been specifically designed to be orthogonal to the human genome. Multiple inducible synZiFTR systems have been developed using clinically approved drugs as the inducer, leading to the first dual inducible gene expression control system in human primary T cells to regulate CAR and cytokine expression (Israni et al., 2021). In addition to clinically approved drugs, natural products, such as the resveratrol found in red wine, grapes, and berries, has also been used to repress or induce CAR expression, demonstrating its applicability in primary T cells, both *in vitro* and *in vivo*, with high dynamic range (Yang et al., 2021).

Inducible gene switches with memory features will allow for long-term changes in gene expression with transient drug exposure. This feature will minimize the need to continuously administer the drug inducer, which can be beneficial when persistent drug administration is impossible or may result in some toxicity. Using a recombinase-based gene circuit with the FlpO-ERT2 fusion protein, drug-inducible CAR expression with memory was developed to induce CAR expression (Chakravarti et al., 2019). Depending on the initial design of the target gene, the circuit can be used to either turn ON or OFF CAR expression.

## Light

Light-inducible dimerization domains have been utilized to make photoactivable CARs in immune cells (Figure 5A) (Tan et al., 2017). A localized CAR expression system in T cells through a blue-light-inducible system was demonstrated previously (Allen et al., 2019; Huang et al., 2020). A similar optogenetic approach was used to induce cytokine expression in T cells for eliminating cancer cells (Zhao et al., 2019). Using a non-invasive light-inducible system, precise spatiotemporal control is possible with minimal side effects, which is difficult to achieve with a small molecule-inducible gene expression system. However, blue light has minimal tissue penetration depth (less than 1 μm), thus limiting its clinical applications. To address this limitation, a nanoplate technology has been developed that can upconvert near-infrared light (NIR), a more transmittable light in tissue, into blue light. By injecting the nanoplate with blue light-inducible CAR T cells into tumor-bearing mice, reversible and real-time control of the CAR activation was achieved to mitigate the potential cytokine storm (Nguyen et al., 2021).

## Ultrasound

Given the challenge of using light as the inducer, ultrasound presents an attractive alternative as a physical inducer, thanks to its safety and greater penetration depth. Pan et al. utilized a mechanically sensitive Piezo1 calcium channel that can be activated by ultrasound (Pan et al., 2018). The exposure to ultrasound creates microbubbles, which activate the Piezo1 channel, enabling calcium intake into the cell. The influx of calcium activates calcineurin, which leads to downstream dephosphorylation of an NFAT transcription factor. The NFAT responsive promoter was used to induce CAR transcription after ultrasound exposure. However, the requirement for microbubbles hinders their application *in vivo*. To circumvent this challenge, the same group of researchers developed a heat-induced CAR that responds to ultrasound (Wu et al., 2021). Focused ultrasound waves increase local temperature, and heat shock protein promoter encoding Cre recombinase can initiate and maintain the CAR expression (Figure 5B).

Miller et al. also implemented a heat-responsive element to control T cell activity (Miller et al., 2021). Instead of directly inducing CAR activity, they used a plasmonic gold nanorod to convert NIR into heat. This system demonstrated successful trafficking of T cells to the antigen-expressing tumor when T cells are expressing CAR. Moreover, they used a plasmonic

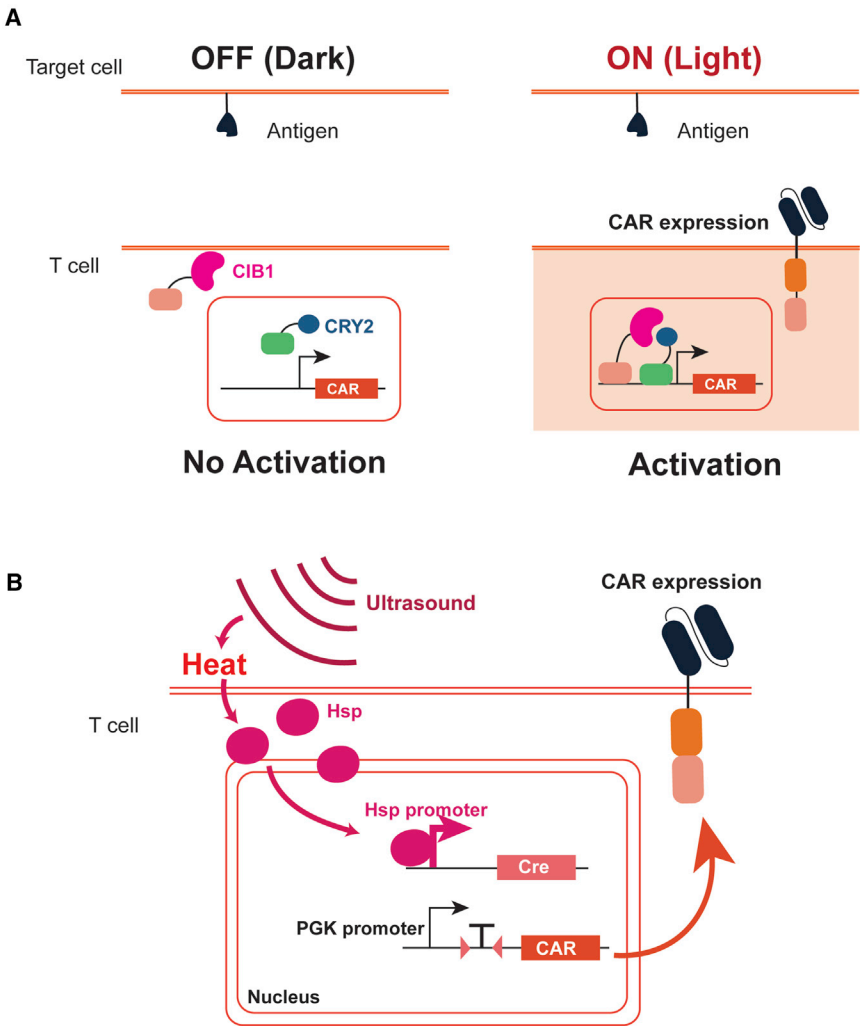


**Figure 5. Exogenous cell control with light and ultrasound inputs**

(A) Photoactivable CAR activation system is designed with two-light-inducible dimerization domains fused to transcription regulatory elements. Upon light induction, the CAR will be expressed and activate the T cells.

(B) Ultrasound-inducible CAR system utilizes the heat shock protein (Hsp)-mediated gene expression. Ultrasound causes heating and increases local temperature, leading to Hsp translocation. Hsp translocation will induce Cre expression, and the Cre will mediate the CAR expression on the T cell.

gold nanorod to convert NIR into heat. They demonstrated that the generated heat could induce both IL-15 superagonist expression to enhance CAR activity *in vivo* and bispecific T cell engager (BiTE) expression to mitigate tumor outgrowth due to antigen escape.

## DISCUSSION

One of the most intriguing features of using cells as therapies is the ability of cells to sense the environment and perform many tasks. As such, developing a strategy for engineering multiple features and functions into cell therapies while also addressing the concerns of safety, specificity, and efficacy would be highly desirable. As highlighted above, many different powerful systems have been developed to approach these challenges. We introduced two arms of immune cell switches: cell-autonomous and cell-exogenous control. The two arms are not mutually exclusive, and they can be used cooperatively. Our ability to rewire receptor machinery and design more ways of controlling the engineered immune cells will further enhance CAR T cell therapies as a whole.

Although many of the ideas discussed here are still in their early stage, some of them are closer to the clinics than others. Currently, logic CARs seem to have the most momentum. For instance, 2-input OR gate CARs have already been evaluated in the clinics and shown promising results (Spiegel et al., 2021). Several companies are also actively pursuing NIMPLY (A AND NOT B) gate CARs for various cancers (Sandberg et al., 2022; Garrison et al., 2021). Many CAR T cell therapies have been designed to also produce factors, such as checkpoint inhibitors (Zhao et al., 2022), immunomodulatory factors (Bell and Gottschalk, 2021; Li and Lim, 2020), or prodrug modifying enzymes (Gardner et al., 2022), to augment the anti-tumor activity. Such designs, while necessary, also heighten the risk of severe adverse side effects. Therefore, regulatable control of CAR activity and transgene expression will be needed to balance activity and safety. Some of the drug-inducible CARs and gene switches described here, especially those that use clinically approved drugs, can provide the safety control needed and therefore are likely to move into clinics in the near future.

A major challenge that hinders the field from realizing its full potential is the ability to perform large-scale genetic engineering on human immune cells, especially cells derived from primary sources. Even with the advancement of pioneering genome editing technologies with CRISPR systems (Kim et al., 2021), the delivery and integration of large DNA payloads represent a major bottleneck that is not readily solvable. An approach to circumvent this complication is to engineer a consortium of immune cells that separately carry the cell-autonomous and cell-exogenous systems, akin to our immune system. These smaller genetic programs could potentially be delivered to T cells *in situ* (Rurik et al., 2022), thus bypassing the need for the complicated *ex vivo* manufacturing process and lowering the cost of the therapy. We envision an ideal scenario where complex genetic circuits with enhanced specificity, efficacy, and safety features are delivered *in situ* into multiple immune cell types, upgrading the patients' immune systems to combat and protect against a myriad of diseases.

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## DECLARATION OF INTERESTS

W.W.W. is a co-founder and shareholder of Senti Biosciences. A.S.K. is a shareholder of Senti Bioscience and Chroma Medicine. S.L. is a current employee of Sanofi.

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