RESEARCH ARTICLE SUMMARY

SYNTHETIC BIOLOGY

Remote control of therapeutic T cells through a small molecule–gated chimeric receptor

Chia-Yung Wu, Kole T. Roybal, Elias M. Puchner, James Onuffer,* Wendell A. Lim*

INTRODUCTION: Cell-based therapies have emerged as a promising treatment modality for diseases such as cancer and autoimmunity. T cells engineered with synthetic receptors known as chimeric antigen receptors (CARs) have proven effective in eliminating chemotherapy-resistant forms of B cell cancers. Such CAR T cells recognize antigens on the surface of tumor cells and eliminate them. However, CAR T cells also have adverse effects, including life-threatening inflammatory side effects associated with their potent immune activity. Risks for severe toxicity present a key challenge to the effective administration of such cell-based therapies on a routine basis.

RATIONALE: Concerns about the potential for severe toxicity of cellular therapeutics primarily stem from a lack of precise control over the activity of the therapeutic cells once they are infused into patients. Exogenously imposed specific regulation over the location, duration, and intensity of the therapeutic activities of engineered cells would therefore be desirable. One way to achieve the intended control is to use small molecules to gate cellular functions. Small molecules with desired pharmacologic properties could be systemically or locally administered at varying dosages to achieve refined temporal and spatial control over engineered therapeutic cells.

RESULTS: We developed an ON-switch CAR that enables small molecule–dependent, titratable, and reversible control over CAR T cell activity. ON-switch CAR T cells required not only a cognate antigen but also a priming small molecule to activate their therapeutic functions. Depending on the amount of small molecule present, ON-switch CAR T cells exhibited titratable therapeutic activity, from undetectable to as strong as that of conventional CAR T cells. The ON-switch CAR was constructed by splitting key signaling and recognition modules into distinct polypeptides appended to small molecule–dependent heterodimerizing domains. The ON-switch CAR design is modular; different antigen recognition domains and small-molecule dimerizing modules can be swapped in.

CONCLUSION: The ON-switch CAR exemplifies a simple and effective strategy to integrate cell-autonomous decision-making (e.g., detection of disease signals) with exogenous, reversible user control. The rearrangement and splitting of key modular components provides a simple strategy for achieving integrated multi-input regulation. This work also highlights the importance of developing optimized bio-inert, orthogonal control agents such as small molecules and light, together with their cellular cognate response components, in order to advance precision-controlled cellular therapeutics. ■

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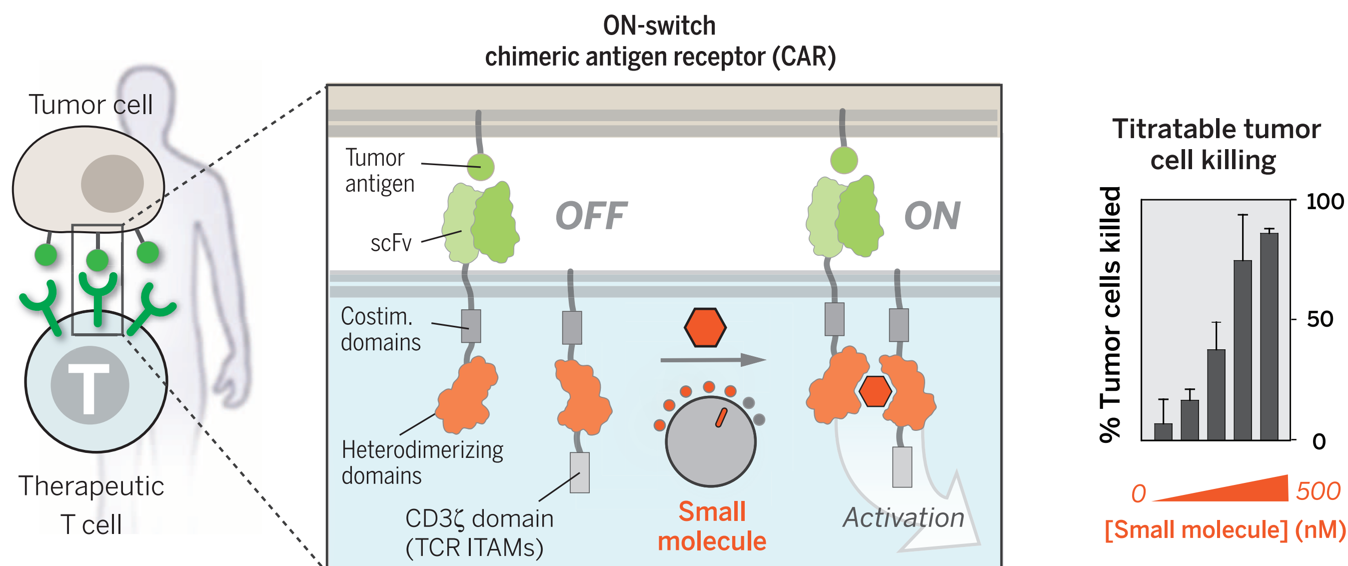
Titratable control of engineered therapeutic T cells through an ON-switch chimeric antigen receptor. A conventional CAR design activates T cells upon target cell engagement but can yield severe toxicity due to excessive immune response. The ON-switch CAR design, which has a split architecture, requires a priming small molecule, in addition to the cognate antigen, to trigger therapeutic functions. The magnitude of responses such as target cell killing can be titrated by varying the dosage of small molecule to mitigate toxicity. scFv, single-chain variable fragment; ITAM, immunoreceptor tyrosine-based activation motif.



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Remote control of therapeutic T cells through a small molecule–gated chimeric receptor

Chia-Yung Wu,[1,2] Kole T. Roybal,[1,2] Elias M. Puchner,[1*] James Onuffer,[1,2]† Wendell A. Lim[1,2,3]†

There is growing interest in using engineered cells as therapeutic agents. For example, synthetic chimeric antigen receptors (CARs) can redirect T cells to recognize and eliminate tumor cells expressing specific antigens. Despite promising clinical results, these engineered T cells can exhibit excessive activity that is difficult to control and can cause severe toxicity. We designed "ON-switch" CARs that enable small-molecule control over T cell therapeutic functions while still retaining antigen specificity. In these split receptors, antigen-binding and intracellular signaling components assemble only in the presence of a heterodimerizing small molecule. This titratable pharmacologic regulation could allow physicians to precisely control the timing, location, and dosage of T cell activity, thereby mitigating toxicity. This work illustrates the potential of combining cellular engineering with orthogonal chemical tools to yield safer therapeutic cells that tightly integrate cell-autonomous recognition and user control.

Cell-based therapies have emerged as promising treatments for a range of disorders, including cancer, autoimmunity, and injury or degeneration (*1–6*). In contrast to small molecules and macromolecules, cellular therapeutic agents have the potential to sense inputs, make decisions, and execute highly complex tasks (*7–9*). A recent example is the use of engineered T cells for adoptive immunotherapy of cancer. Primary T cells can be isolated and engineered to express synthetic chimeric antigen receptors (CARs)—receptors that combine an extracellular, single-chain antibody domain, which recognizes a specific tumor-associated antigen, with intracellular signaling domains from the T cell receptor (TCR) and costimulatory receptors (*2–4*, *10*). In clinical trials, CAR T cells directed against the B cell antigen CD19 have proven effective against chemotherapy-resistant forms of B cell cancers (*11–15*). Upon antigen ligand engagement, CAR T cells execute multiple key therapeutic functions, including production of antitumor cytokines and killing of target tumor cells (Fig. 1A). Antigen binding also stimulates exponential proliferation of the therapeutic T cells in vivo. Infused CAR T cells can expand by a factor of >1000, resulting in a highly amplified response and consequent eradication of a large number of tumor cells within weeks (*11*).

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Such cell-based therapies, however, can also be associated with severe toxicities (Fig. 1A). Off-tumor cross-reaction of engineered T cells can lead to killing of nontumor cells. If such cross-reaction occurs to cells in the heart, lung, or liver, then the high doses of CAR T cells that these tissues are exposed to upon initial cell injection can lead to rapid death (*16*). Even with successful tumor targeting, the rapid rise in the overall T cell activity fueled by CAR signaling during treatment can also lead to systemic life-threatening side effects, such as those caused by release of excessive cytokines. Rapid elimination of large numbers of tumor cells in a short time frame also can result in tumor lysis syndrome. Both conditions can trigger multi-organ failure and require urgent medical intervention. Patient-to-patient variations in T cell responses and in risks for toxicity make it challenging to predict the optimal number of T cells to infuse (*2*). Thus, the engineering of regulatory systems that allow for control over the dose and timing of T cell function is an important priority.

One approach is to engineer suicide switches to eliminate the infused T cells if their toxic effects begin to get out of control (*17*, *18*). Examples include a small molecule–regulated caspase that triggers apoptosis of the T cells (Fig. 1B). Another approach is to engineer negative regulatory co-receptors that can override killing responses when a specific "do not kill" ligand is recognized (*19*). Although these strategies are important elements in the toolbox for engineering therapeutic T cells, they have several drawbacks. The suicide switches irreversibly abort the complex and expensive treatment, and they may not act fast enough to prevent cross-reaction during initial cell transfer. Overriding inhibitory co-receptors can prevent killing of particular cells that express a specific ligand, but they cannot control the timing and intensity of T cell activity more generally.

We therefore aimed to develop a complementary strategy for controlling CAR T cells that focused instead on positive regulation, in which an exogenous, user-provided signal such as a small molecule is required for activation (Fig. 1B). This kind of "ON-switch" would complement the other classes of control systems and provide important advantages. Positive regulation could allow for gradual titration of activity to appropriate therapeutic levels, as well as control of the timing of activation, thus preventing first-pass toxicities that could occur immediately upon cell transplantation. Coupled to technologies for localized and sustained delivery of small molecules (*20*), an ON-switch could impart spatial control over the therapeutic effects to mitigate off-tumor toxicities. Several features would be important for an ON-switch CAR design: (i) The receptor would still need to be dependent on specific tumor antigen recognition for T cell activation; small molecule alone or antigen alone should not activate. (ii) The therapeutic activity of the T cell population should be titratable by varying the concentration of the small molecule, and at high enough levels this activity should be comparable to that of conventional CAR T cells. (iii) The timing of the CAR T cell response should be reversibly controllable by addition or removal of the small molecule.

The engineering goal for an ON-switch CAR embodies a general problem that evolution has repeatedly faced: how to convert a signaling molecule that is gated by a single input into a molecule that is combinatorially gated by two inputs (in this case, a receptor that requires both an antigen and a small molecule for T cell activation). Natural signaling systems often achieve tight control over critical processes through this type of combinatorial regulation. The general ability to engineer synthetic receptors that function as Boolean AND-gates responding to two inputs—one an autonomously recognized disease signal (i.e., tumor antigen), and the other a user-controlled signal (i.e., small molecule)—would be broadly useful for engineering safe cellular therapeutic agents of any kind.

One can imagine highly complex regulatory mechanisms involving precise conformational allostery that could yield a signaling receptor that functions as a Boolean AND gate. However, very often, controls that have evolved in living systems use simpler strategies such as controlled assembly: An active molecular system can be split into multiple parts, such that it is dependent on multiple inputs that promote assembly of the intact molecular system (*21*). Thus, we focused on constructing split synthetic receptor systems in which the assembly of an activated complex was dependent on both binding of a small molecule and antigen engagement (Fig. 1C). The concept of splitting key components from the CAR is itself inspired by the natural process of T cell activation, which normally requires the coengagement of the T cell receptor (by peptide–major histocompatibility complex)

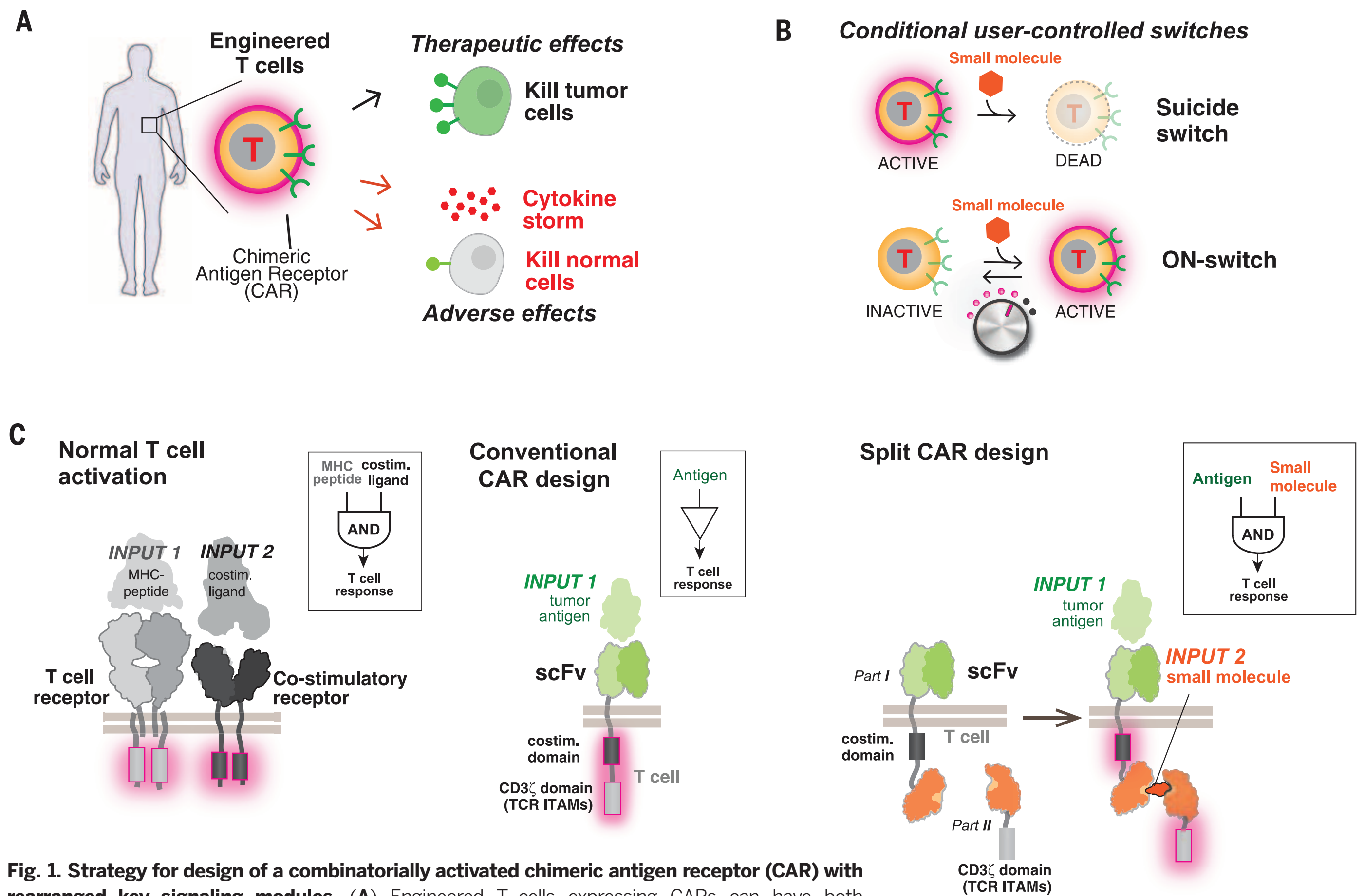


Fig. 1. Strategy for design of a combinatorially activated chimeric antigen receptor (CAR) with rearranged key signaling modules. (**A**) Engineered T cells expressing CARs can have both therapeutic and adverse effects. (**B**) User-controlled switches. A suicide switch triggers apoptosis of the engineered cells. An alternative, complementary approach is keeping the cells inactive until addition of an activating small-molecule drug signal. Such an ON-switch could allow for titratable control (dialing up or down) of T cell activity. (**C**) Molecular strategies to control T cell activation. The normal T cell activation pathway (left) entails dual activation of the TCR and a costimulatory receptor to trigger key cellular responses such as cytokine production and proliferation. The conventional CAR (center) combines an antigen recognition domain (scFv) with main signaling motifs (such as ITAMs from TCR subunit CD3ζ) and costimulatory motifs constitutively linked in a single molecule. A strategy for constructing a split CAR design (right) distributes key components from the conventional CAR into two physically separate polypeptides that can be conditionally reassembled when a heterodimerizing small-molecule agent is present. The design resembles an AND logic gate that requires "antigen + small molecule" combinatorial inputs for T cell activation.

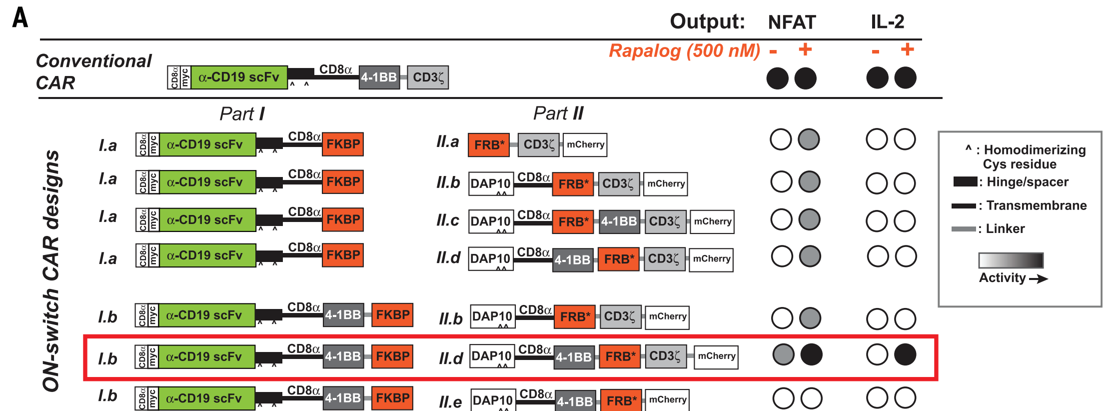
and costimulatory receptors (e.g., 4-1BB or CD28) (Fig. 1C). The conventional CAR represents a construct in which components from the TCR and costimulatory receptors are artificially colocalized. Thus, our strategy represents resplitting key signaling modules in a different, small molecule–controlled configuration.

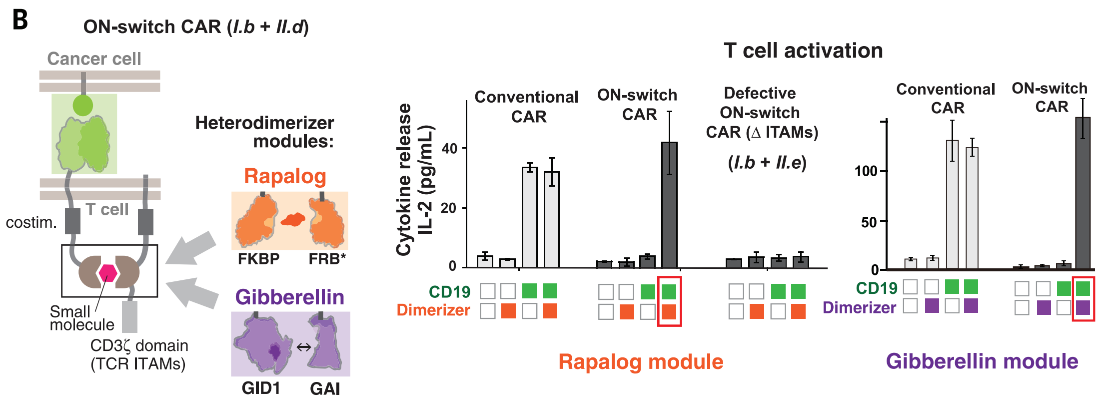
We used this strategy to engineer a robust ON-switch CAR design that fulfills the criteria of yielding titratable, reversible, and temporarily controllable activity in CAR T cell populations. This work provides a general strategy for how to engineer dual-input synthetic receptors that require a small molecule as a coactivation signal. The ON-switch CAR framework also provides an important tool for developing the next generation of precision-controlled therapeutic T cells.

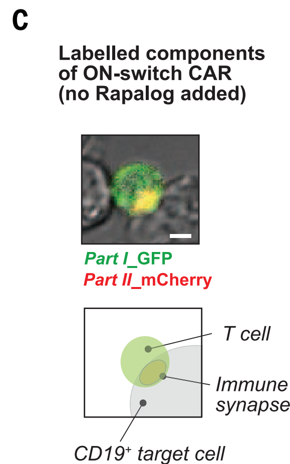
Design of ON-switch CARs

To construct a CAR that required both an antigen AND a small molecule for activation, we used a split-receptor design that structurally resembles natural immune receptors such as the B and T cell receptors (BCRs and TCRs), whose antigen-binding and intracellular signaling domains are found on separately expressed polypeptides. In these natural cases, heterodimerization of these distinct polypeptides is required to assemble a functioning receptor complex (22). This type of multipolypeptide assembly presents the opportunity to engineer receptor control by enforcing small-molecule dependence on this assembly. The envisioned ON-switch CAR (Fig. 1C) consists of two parts that assemble in a small molecule–dependent manner. Part I of the receptor features an extracellular antigen-binding domain [a single-chain variable fragment (scFv)]. Part II has a key downstream signaling element: the immunoreceptor tyrosine-based activation motifs (ITAMs) from the T cell receptor CD3ζ subunit (22). The ITAM motifs are phosphorylated upon T cell receptor activation, resulting in the recruitment of Src homology 2 (SH2) domain effectors (such as the kinase ZAP70) and triggering the cascade of T cell activation. The two parts of the split receptor contain heterodimerization domains that conditionally interact upon binding of a heterodimerizing small molecule. Particular cellular responses, including T cell activation, have been engineered to be triggered solely by small molecule–induced dimerization (23, 24). Our goal, in contrast, was to design a new class of CAR whose small molecule–induced assembly is necessary but not sufficient for cellular activation. The small molecule thus acts as a priming or licensing factor that is a precondition for antigen-triggered activation.

We explored multiple ways of splitting components of the conventional CAR molecule to find a configuration that would strongly impair its activity but still allow for strong antigen-induced signaling when the receptor components were assembled in the presence of the small molecule (Fig. 2A). To facilitate the design of a prototype,







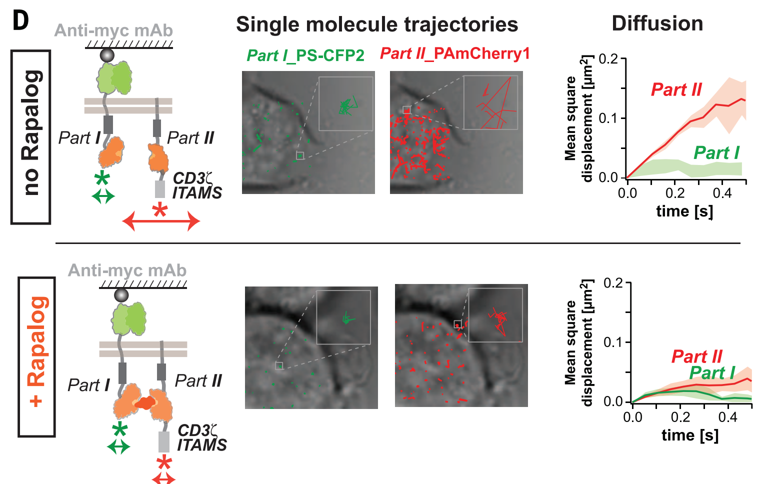


Fig. 2. Construction and screening of an ON-switch CAR that is dependent on the presence of a small-molecule dimerizer. (**A**) ON-switch CAR candidate constructs and their functional behavior. Candidate construct pairs were expressed in Jurkat T cells. Cells were incubated with K562 target cells expressing the cognate antigen CD19+ in the presence or absence of 500 nM rapalog. Activation was quantified via expression of a NFAT-dependent GFP reporter gene and production of the cytokine IL-2. The part I constructs of the ON-switch CAR share many features with the conventional CAR: the CD8α signal sequence, a Myc epitope, the anti-CD19 scFv, and the CD8α hinge and transmembrane domain, in addition to the FKBP domain for heterodimerization. The part II constructs consisted of the T cell receptor CD3ζ signaling chain that is critical for T cell activation, the FRB* domain for heterodimerization, and the mCherry tag. More advanced part II variants contained the additional DAP10 ectodomain for homodimerization and the CD8α transmembrane domain for membrane anchoring. The 4-1BB costimulatory motif was inserted in various locations, depending on the construct. The best ON-switch construct (I.b + II.d) is outlined in red. (**B**) Response of ON-switch CAR (I.b + II.d) to rapalog and antigen stimulation. Jurkat cells expressing the specified CARs were incubated with K562 target cells expressing either the cognate antigen (CD19; green squares) or a noncognate antigen (mesothelin; white squares). Presence of 500 nM rapalog in the sample is indicated by orange squares. Production of IL-2 after an overnight incubation was quantified by enzyme-linked immunosorbent assay (ELISA); *n* = 3, error bars denote SD. Similar results were observed for ON-switch CARs in which the rapalog heterodimerization module was replaced by an alternative module, the gibberellic acid (GA) heterodimerization module (using *Arabidopsis* GID1 and GAI domains). The ON-switch CAR with GA dimerizing domains requires both cognate antigen and GA (purple squares) to trigger cytokine production. (**C**) The ON-switch CAR components colocalize in the absence of dimerizing rapalog. Parts I and II of the receptor are labeled with GFP and mCherry, respectively. The confocal microscopy images are pseudo-colored to indicate localization of both parts. Image shows a primary human CD8+ T cell expressing the anti-CD19 ON-switch CAR engaged with a CD19+ K562 target cell in the absence of rapalog. Scale bar, 5 μm. (**D**) Two-color single-molecule tracking shows independent movement of ON-switch CAR components in the absence of rapalog. Left panels: Jurkat T cells were adhered to a cover slip coated with an antibody to the Myc epitope in order to immobilize the receptors (extracellular region of part I CAR is tagged with Myc). Individual parts of the ON-switch CAR were each tagged with photoactivatable fluorescent proteins PS-CFP2 and PAmCherry1. Center panels: The single-molecule trajectories of part I (green) and part II (red) are superimposed on transmitted light images of the cells (gray). Right panels: The average mean-square displacement of trajectories quantifies the diffusive behavior (solid lines, average from multiple cells; colored band, SD to represent cell-cell variability). Part I molecules are immobile because of antibody tethering, whereas in the absence of rapalog (top), part II molecules exhibited fast diffusion. In the presence of 500 nM rapalog (bottom), however, the part II molecules became immobile, confirming rapalog-induced assembly of the two-component receptor.

we used a set of structurally well-defined heterodimerizing components: the FK506 binding protein (FKBP) domain and the T2089L mutant of FKBP-rapamycin binding domain (FRB*) that heterodimerize in the presence of the rapamycin analog AP21967, which has less immunosuppressive activity than does rapamycin (25–27). We refer to this modified rapamycin as the rapalog. We screened candidate receptors for rapalog-dependent activation in the human $CD4^+$ Jurkat T cell line with two assays. We assayed the activity of a synthetic promoter composed of multiple copies of NFAT (nuclear factor of activated T cells) response elements (28), a highly sensitive readout for T cell receptor activation. We also measured interleukin-2 (IL-2) cytokine secretion, which represents a more stringent, integrated cellular response. The simplest split ON-switch design examined, comprising constructs I.a + II.a (Fig. 2A), consisted of a cytoplasmic ITAM fragment that could be recruited to an antigen-binding membrane receptor component upon addition of rapalog. This initial receptor design failed to signal strongly in either assay, despite abundant expression of both receptor parts in Jurkat cells (figs. S1 to S3).

We suspected that the entropic cost for driving a cytoplasmic fragment to bind its membrane-associated partner might be too high for this small molecule–induced interaction. Thus, we explored localization of both receptor parts at the plasma membrane. We designed constructs that targeted both fragments to the plasma membrane but varied the domain composition and order within both parts of the CAR (Fig. 2A). We targeted part II to the plasma membrane by appending the same CD8α transmembrane domain used in part I. We also appended the ectodomain of DNAX-activating protein 10 (DAP10) to part II. The DAP10 ectodomain mediates homodimerization (29), effectively doubling the copy number of ITAMs per part II molecule. This modification was expected to increase receptor output activity, as the copy number of ITAMs correlates positively with receptor signaling strength (30, 31). We also varied the positioning of the 4-1BB costimulatory domain, which promotes T cell proliferation and survival, in both parts I and II. Previous work suggests that the 4-1BB costimulatory domain functions best when placed adjacent to the plasma membrane (32). We also varied the position of the FKBP and FRB* heterodimerizing domains. These changes led to the design of constructs I.b, II.b, II.c, and II.d (Fig. 2A). Molecule II.e, which lacks the CD3ζ sequence, was constructed as a corresponding negative control (defective ON-switch CAR).

Identification of a dual input–gated ON-switch CAR construct

The most promising design from this set of components comprised the components I.b and II.d (Fig. 2A). When stimulated by target cells expressing the cognate antigen (CD19) in the presence of 500 nM rapalog, this combination led to strong cytokine production comparable to that stimulated by the conventional single-component CAR. The response was highly small molecule–dependent. This was the only split-receptor design within this subset that signaled as strongly as did the conventional CAR (fig. S3). Further evaluation confirmed that neither the CD19 cognate antigen nor the small molecule alone was sufficient to trigger IL-2 production (Fig. 2B). These results in the Jurkat T cell line indicate the successful construction of an AND-gate receptor requiring dual inputs.

ON-switch CAR architecture is compatible with alternative heterodimerization modules and alternative antigen-binding domains

We tested whether this ON-switch CAR architecture would show similar dual-input regulation with different small-molecule heterodimerization domains or with different antigen recognition domains. We used the gibberellin-induced dimerization system GID1-GAI (Fig. 2B) (33). Derived from plants and structurally unrelated to FKBP-FRB* (27, 34), this alternative small-molecule input system yielded an ON-switch CAR population that was inactive in the absence of gibberellin but became activated when stimulated by both antigen (CD19) and gibberellin. This alternative system worked equally well to gate T cell activation (Fig. 2B). Thus, the ON-switch CAR design appears to work with alternative heterodimerization systems.

To test whether the design would function with alternative antigen-binding domains, we reconstructed the system with a single-chain antibody that recognizes the antigen mesothelin instead of the antigen CD19. Jurkat T cells expressing this version of the receptor maintained dual input control, requiring both stimulation with mesothelin-expressing target cells and addition of rapalog (fig. S4).

Single-molecule imaging shows that two components of the ON-switch CAR assemble only in the presence of small-molecule dimerizer

The ON-switch CAR was designed to conditionally assemble only in the presence of rapalog. To determine the localization of the two parts and how this changed with the addition of rapalog, we labeled part I with green fluorescent protein (GFP) and part II with mCherry. When we mixed the ON-switch CAR T cells with cognate antigen-expressing cells in the absence of rapalog, the T cells formed stable cell-cell junctions (immune synapses) with the target cells. Both part I and part II of the ON-switch CAR localized at the synapse, even in the absence of rapalog. Fluorescence microscopy of these components revealed overlapping localization (Fig. 2C).

To better understand how the interaction of the two molecular parts changes with addition of rapalog, we used photoactivated localization microscopy (PALM) (35–37). This type of imaging can yield critical information about the location and dynamics of the individual molecules. We expressed the ON-switch CAR in Jurkat cells, with each component labeled with a distinct photoactivatable fluorescent protein [part I was labeled with photoswitchable cyan fluorescent protein 2 (PS-CFP2) and part II with photoactivatable mCherry1 (PAmCherry1)]. Cells were placed on glass slides coated with an antibody that binds to a Myc epitope tag placed at the N terminus (extracellular region) of part I of the receptor (Fig. 2D, left). Interaction with the antibody effectively immobilized the cells and anchored the part I molecules to the slide surface (fig. S5). The distribution of individual part I and part II molecules at the slide interface was then traced over time by tracking the photoactivatable fluorescent protein tags.

We observed small, constrained dynamic trajectories for part I molecules, confirming the immobility of this component upon antibody tethering. In contrast, the large, unconstrained dynamic trajectories of the part II molecules indicated fast diffusion of this component in the absence of rapalog (Fig. 2D, top, and movie S1). The measured average diffusion coefficient of ~0.1 $\mu m^2/s$ observed for the part II molecules is well within the range expected for unconstrained transmembrane proteins (38). The major change that we observed upon addition of rapalog was that the part II molecules also became immobile; their trajectories decreased to match those of the antibody-tethered part I molecules (Fig. 2D, bottom, and movie S2).

Thus, although both components of the ON-switch CAR appeared to be colocalized at a macroscopic level in the absence of the dimerizing small molecule, at the single-molecule level the two components were not appreciably physically associated. Without the dimerizing small molecule, the part II molecules diffused within the membrane in an unconstrained manner, even though the part I molecules were firmly anchored to an extracellular antibody. However, upon addition of the small molecule, the diffusion of part II became equally constrained, consistent with a model in which the two parts tightly associate only in the presence of the dimerizing small molecule. These findings are consistent with a model in which a critical part of the switch design is the localization of both parts of the receptor to the plasma membrane. The interaction mediated by small-molecule addition is sufficient to drive a large change in the molecular association of the two parts.

ON-switch CAR requires small molecule and antigen to activate primary human $CD4^+$ T cells

We tested the effectiveness of this ON-switch CAR (I.b + II.d; the rapalog-controlled version) in therapeutically relevant T cells. We expressed the components in primary human $CD4^+$ helper T cells and examined multiple T cell responses (Fig. 3A). Expression of the ON-switch CAR was similar to that of the conventional CAR in primary T cells (fig. S6). CAR T cells were incubated with antigen-expressing target cells in varying concentrations of rapalog. All of the tested $CD4^+$ T cell responses showed the dual requirement for stimulation with cognate antigen ($CD19^+$ target cells) and small-molecule dimerizer. These responses included IL-2 production (a general readout for effector T cell activation) and interferon-γ

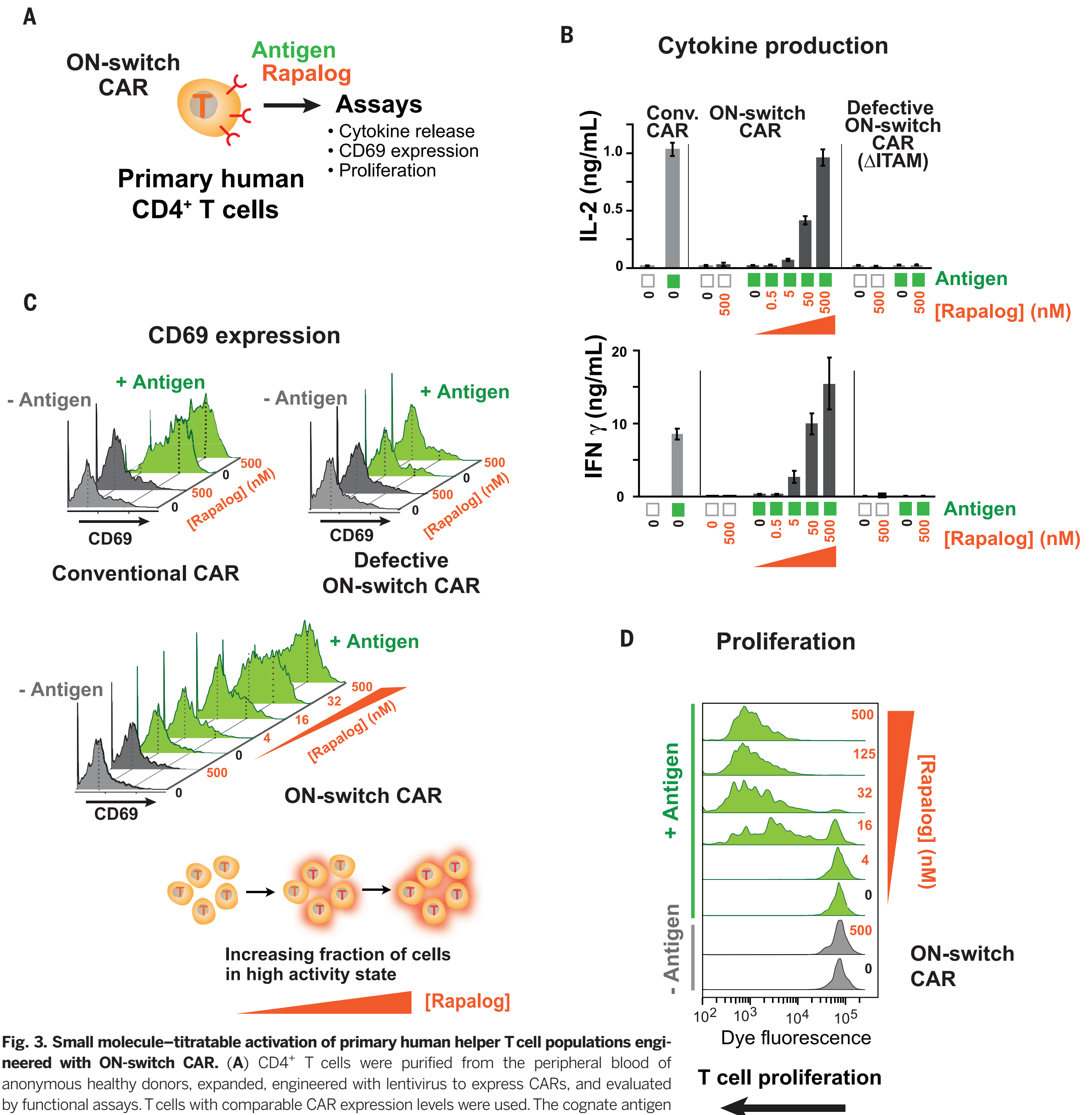


Fig. 3. Small molecule–titratable activation of primary human helper T cell populations engineered with ON-switch CAR. (**A**) CD4+ T cells were purified from the peripheral blood of anonymous healthy donors, expanded, engineered with lentivirus to express CARs, and evaluated by functional assays. T cells with comparable CAR expression levels were used. The cognate antigen CD19 was presented to T cells as a cell surface protein on K562 target cells. Various concentrations of the dimerizer rapalog were added to reaction mixtures to examine effects of rapalog titration. (**B**) Production of the cytokines IL-2 and IFN-γ quantified by ELISA after an overnight incubation, as described in supplementary material; *n* = 3, error bars denote SD. (**C**) Monitoring T cell activation in single cells by quantifying expression of the cell surface protein CD69, whose up-regulation occurs early during T cell activation. T cells in overnight assay mixtures were stained with a fluorophore-conjugated antibody to CD69 and analyzed by flow cytometry. Green histograms denote T cells stimulated with CD19+ target cells (+ antigen). Gray peaks denote T cells treated with target cells lacking the CD19 antigen (– antigen). T cell population shows bimodal response, and addition of rapalog increases the fraction of cells in the high-response population. (**D**) Dimerizer small molecule– and antigen-dependent T cell proliferation. T cells expressing the ON-switch CAR were prelabeled with the intracellular dye CellTrace Violet, whose fluorescence intensity per cell progressively decreases with increasing rounds of cell division. Cells were processed in a flow cytometer after 5 days of incubation. Leftward shift of peaks in the histogram indicates T cell proliferation.

(IFN-γ) production [an indicator for T helper cell 1 (T_H1) antitumor response]. Production of IL-2 and IFN-γ was minimal when cells were stimulated by either the small-molecule dimerizer or the cognate antigen individually. Dual stimulation with the cognate antigen and increasing amounts of small molecule led to a dose-dependent increase in cytokine secretion by the ON-switch CAR T cells. Notably, at high concentrations of

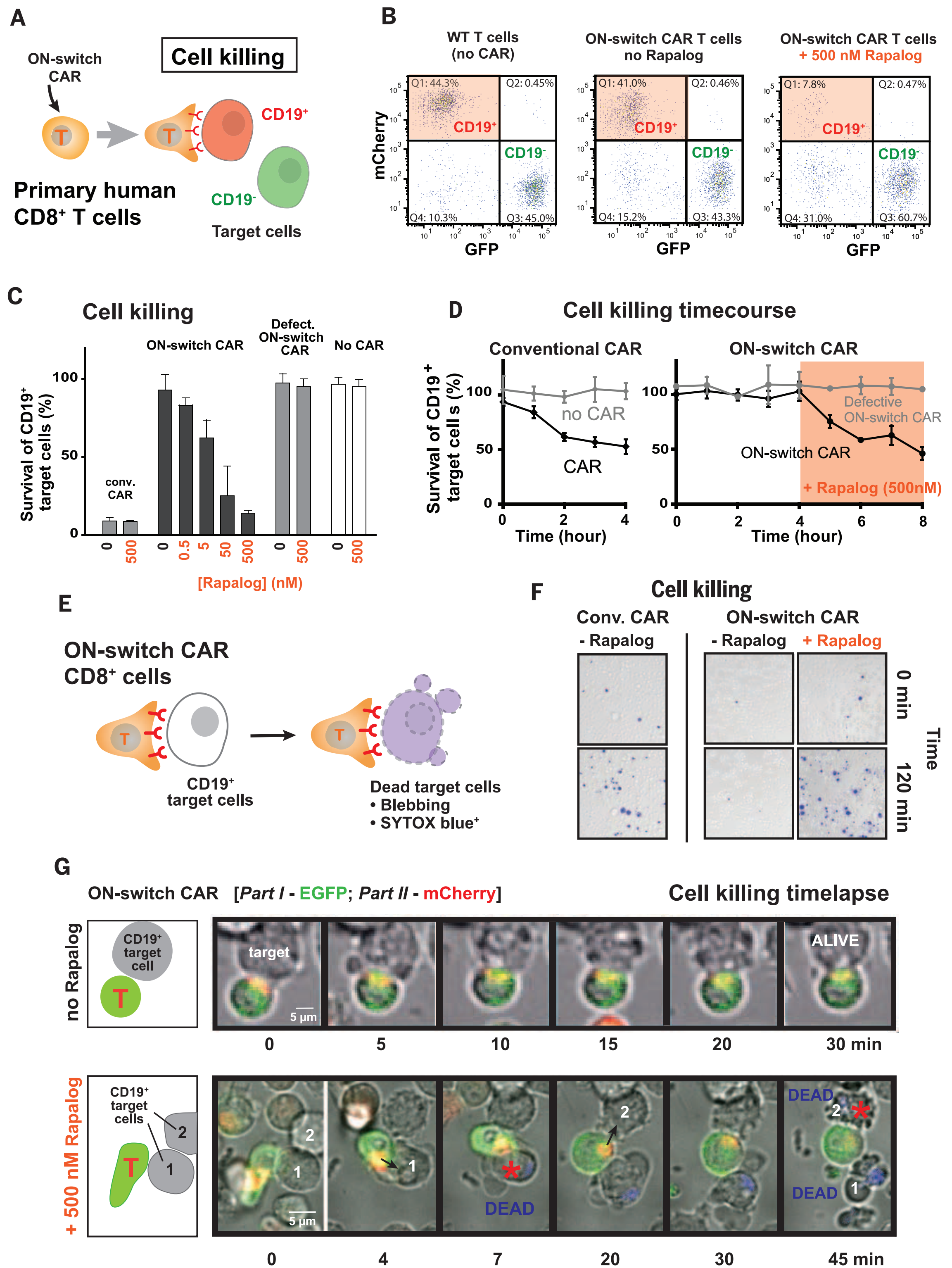


Fig. 4. ON-switch CAR yields antigen-specific and titratable killing of target cell population by engineered primary cytotoxic (CD8^{+}) T cells. (**A**) Schematic of a flow cytometry–based cell-killing assay. Primary human CD8^{+} T cells were isolated, expanded, and engineered to express CARs by transduction with lentivirus. T cells with comparable CAR expression levels were used. T cells were incubated with a mixture of cognate target cells (CD19^{+}, mCherry^{+}) and noncognate target cells (CD19^{-}, GFP^{+}). Rapalog was added to specified concentrations. After incubation for a designated period of time, the abundance of both types of K562 target cells within the overall surviving target cell population was quantified by flow cytometry. (**B**) Representative flow cytometry data. Surviving target cells in sample mixtures at the end of an overnight assay were segregated into cognate (mCherry^{+}) and noncognate (GFP^{+}) subpopulations. The percentage of CD19^{+} cells (quadrant 1) was divided by that of CD19^{-} cells (quadrant 3) to calculate the normalized percentage of survival of cognate target cells in each sample. (**C**) Cytotoxicity mediated by CARs in an overnight (22 hours) end-point experiment. A low percentage for survival of cognate target cells indicates a high degree of specific target cell killing by CAR T cells. (**D**) Cytotoxicity mediated by CARs in a kinetic experiment. Target cell killing by conventional CAR was quantified hourly during a 4-hour incubation period. Cytotoxic activities of the ON-switch CAR were first monitored in the absence of dimerizing small molecule hourly for 4 hours, followed by four more hourly time points in the presence of small molecule (500 nM rapalog). (**E**) Schematic of the experimental setup of the time-lapse imaging experiments. (**F**) Representative differential interference contrast (DIC) images of primary human CD8^{+} T cells expressing the conventional CAR or the ON-switch CAR (± rapalog) incubated with CD19^{+} K562 target cells, overlaid with SYTOX blue dead stain fluorescent images, to assay target cell death after 0 and 2 hours of interaction (n = 3). (**G**) A time-lapse montage of DIC and fluorescence image overlays of primary human CD8^{+} T cells expressing ON-switch CAR (part I tagged with EGFP, part II tagged with mCherry) and their interaction with CD19^{+} K562 targets. The top montage is in the absence of rapalog and shows T cell binding, but no killing of target cells, over the course of the 30-min experiment (movie S3). The bottom montage is in the presence of 500 nM rapalog and shows killing of tumor cells, indicated by blebbing and SYTOX blue dye uptake, within 45 min (movie S4).

dimerizing molecule, cytokine production rivaled that obtained with T cells expressing the conventional CAR (Fig. 3B).

Similarly, dual-gated and titratable responses were seen for expression of the induced cell surface marker CD69. Increased cell surface expression of the CD69 protein occurs within hours after T cell activation and provides a standard method to monitor the activation status of T cells at the single-cell level by flow cytometry (*39*). As was observed with the conventional CAR T cells, cells expressing the ON-switch CAR displayed a bimodal CD69 expression pattern upon activation. However, unlike the conventional CAR that only required the cognate CD19 antigen to activate T cells, the ON-switch CAR also required the dimerizer molecule to be present (Fig. 3C). Increasing dimerizer concentrations did not appear to titrate the activity level of individual cells, but rather increased the fraction of cells in the $CD69^{high}$ activated state.

ON-switch CAR T cells require both antigen and small-molecule dimerizer to drive cell proliferation

Antigen-induced proliferation of CAR T cells is a critical facet of therapeutic responses (*31*, *39*–*41*). Proliferation allows for amplification of T cell action, but excessive proliferation allows for systemic and severe toxicities. We thus tested whether the ON-switch CAR T cells displayed combinatorially gated cell proliferation (Fig. 3D). In a flow cytometry experiment, we labeled human primary $CD4^+$ T cells with an intracellular fluorescent dye that is progressively diluted with increasing rounds of cell division, allowing us to monitor cell proliferation. Proliferation of these cells indeed required both the cognate antigen and rapalog. The observed small molecule–gated control over T cell proliferation is important; controlling the degree of cell expansion in vivo may be a highly effective way to tune and optimize the strength of the therapeutic response.

Our results with primary human $CD4^+$ helper T cells confirm that the ON-switch CAR enables the key therapeutic behaviors of CAR T cells to be gated and regulated by a small-molecule drug. At the single-cell level, our flow cytometry data (for CD69 surface expression and proliferation) indicate that a small molecule–dependent bistable switch alters the number of cells in the ON versus OFF state in the presence of cognate antigen. On the population level, the ON-switch design additionally functions as a dial that yields a titratable dosage of key antitumor responses. The ON-switch CAR T cell populations displayed a high dynamic range of signaling, with a maximum response (at saturating concentrations of small-molecule dimerizer) comparable to that of the conventional CAR T cells.

Small molecule–controlled cancer cell killing by ON-switch CAR $CD8^+$ T cells

We tested the ON-switch CAR's ability to control tumor cell killing by primary human $CD8^+$ cytotoxic T cells. Targeted apoptosis of tumor cells is one of the hallmarks of antitumor immunity and CAR T cell action. A major purpose of CARs is to redirect $CD8^+$ T cell cytotoxicity selectively toward cancer cells expressing antigens of interest. Relative to cytokine production and proliferation, T cell–mediated cell killing occurs on a faster time scale (minutes) and with different thresholds (*42*, *43*). Thus, we examined whether primary human $CD8^+$ T cells expressing the ON-switch CAR could mount a cytotoxic response that was still antigen-specific but gated by the dimerizing small molecule. Control over cytotoxic activity is one of the most important needs for CAR T cell regulation.

To test cell killing, we labeled target cells expressing either the cognate antigen ($CD19^+$) or a noncognate antigen ($mesothelin^+$) by the selective expression of distinct fluorescent proteins, such that in a mixed population the two target cell types could be independently identified and tracked by flow cytometry (Fig. 4A and fig. S7A). The cytotoxic activity of $CD8^+$ T cells expressing the CD19-directed ON-switch CAR was then quantified according to reduction in the fraction of cognate target cells among all viable target cells (Fig. 4B). After an overnight incubation (22 hours), no cell-mediated cytotoxicity was observed in the absence of rapalog. When 500 nM rapalog was added, efficient killing of the cognate target cells was observed. Killing of noncognate target cells was not observed, and the degree of cognate target cell killing could be titrated by changing the concentration of rapalog (Fig. 4C and fig. S7A). The degree of targeted cell killing with saturating rapalog matched the level observed with the conventional CAR.

In an 8-hour time course experiment, T cells expressing the conventional CAR immediately induced specific cytotoxicity in cognate target cells. T cells expressing the ON-switch CAR did not commence killing until addition of rapalog (Fig. 4D). These results confirmed that the ON-switch CAR T cells allow for titratable control over the magnitude and timing of apoptosis in the target cell population. We next tested whether the ON-switch CAR T cells would stop cell killing after rapalog removal and then resume killing upon reintroduction of rapalog. This coculturing experiment consisted of three stages to implement the "on-off-on" sequence of exposure to rapalog, and each stage lasted 36 hours (fig. S8A). Target cell survival was quantified at the beginning and the end of each stage. Target cell killing was only detectable in the first and last 36-hour periods when the rapalog was present (fig. S8, B and C). Our proof-of-principle experiments with $CD8^+$ T cells illustrate that the ON-switch design provides a flexible platform for temporal control over CAR T cell action. In therapeutic settings, the kinetics of on-off and off-on transitions could in principle be tuned with small molecules with different pharmacodynamics, or with competitive (nondimerizing) antagonist molecules.

To confirm the relevance of our observations made with engineered K562 (human chronic myelogenous leukemia) target cell lines, we investigated rapalog-gated killing of natural $CD19^+$ cancer cells by ON-switch CAR T cells. The Raji and Daudi human B cell lines both naturally express CD19 in amounts greater than that in the $CD19^+$ K562 cell line we had used (fig. S7B). Cytotoxicity experiments showed that both B cell lines were subject to killing by $CD8^+$ ON-switch CAR T cells in a manner dependent on the dose of the rapalog (fig. S7C). In summary, we observed titratable killing of multiple types of $CD19^+$ target cell populations.

Imaging of ON-switch CAR T cell killing

To better characterize the spatiotemporal aspects of ON-switch CAR–mediated killing, we performed microscopy-based assays. We quantitatively monitored cell killing by $CD8^+$ T cells by means of SYTOX nucleic acid binding dye, which only permeates dead cells (Fig. 4E). Images taken after 2 hours confirmed killing of $CD19^+$ target cells by the ON-switch CAR T cells, but only in the presence of the small molecule (Fig. 4F).

We directly followed the dynamics of cell death with live-cell time-lapse confocal microscopy. In primary human $CD8^+$ T cells, we expressed the ON-switch CAR components labeled with distinct fluorescent proteins (part I comprising the extracellular scFv domain was labeled with enhanced GFP at the C terminus; part II comprising the CD3ζ domain was labeled with mCherry at the C terminus). We tracked the physical interaction between T cells and cognate target cells as well as the killing of cognate target cells. In the absence of rapalog, T cells and target cells associated and formed an immune synapse–like structure, but target cells remained alive for the duration of the 30-min experiment (movie S3; Fig. 4G, top). Quantitative analysis of time-lapse images revealed that even in the absence of rapalog, ON-switch CAR T cells formed conjugates with cognate target cells as frequently as did conventional CAR T cells (fig. S9). However, the physical association alone did not induce target cell death. In contrast, in the presence of rapalog, multiple target cells were rapidly killed within the 45-min time window (movie S4; Fig. 4G, bottom).

In vivo small-molecule regulation of cognate tumor cell killing

We tested whether the tumor cell killing by primary ON-switch CAR T cells could be regulated in vivo in a mouse xenograft model. In preliminary pharmacokinetic studies, rapalog had a plasma half-life in mice of approximately 4 hours. This property necessitated multiple injections of rapalog per day (three injections at 3 mg/kg to maintain 50 ng/ml in plasma), which, combined with the high cost of the compound, precluded the performance of extended multiweek studies with statistically sufficient numbers of mice. Thus, we used a shorter protocol of in vivo tumor killing (Fig. 5A). We implanted a mixture of cognate ($CD19^+$ $mCherry^+$) and noncognate ($CD19^-$ GFP^{high}) K562 cells into the peritoneal cavity. Fourteen hours later, CAR-expressing $CD8^+$ T cells and the first dose of rapalog (or vehicle control) were injected intraperitoneally (i.p.). Three additional doses of the rapalog or vehicle were injected at 6-hour intervals. Mice were euthanized at the experimental end point (after

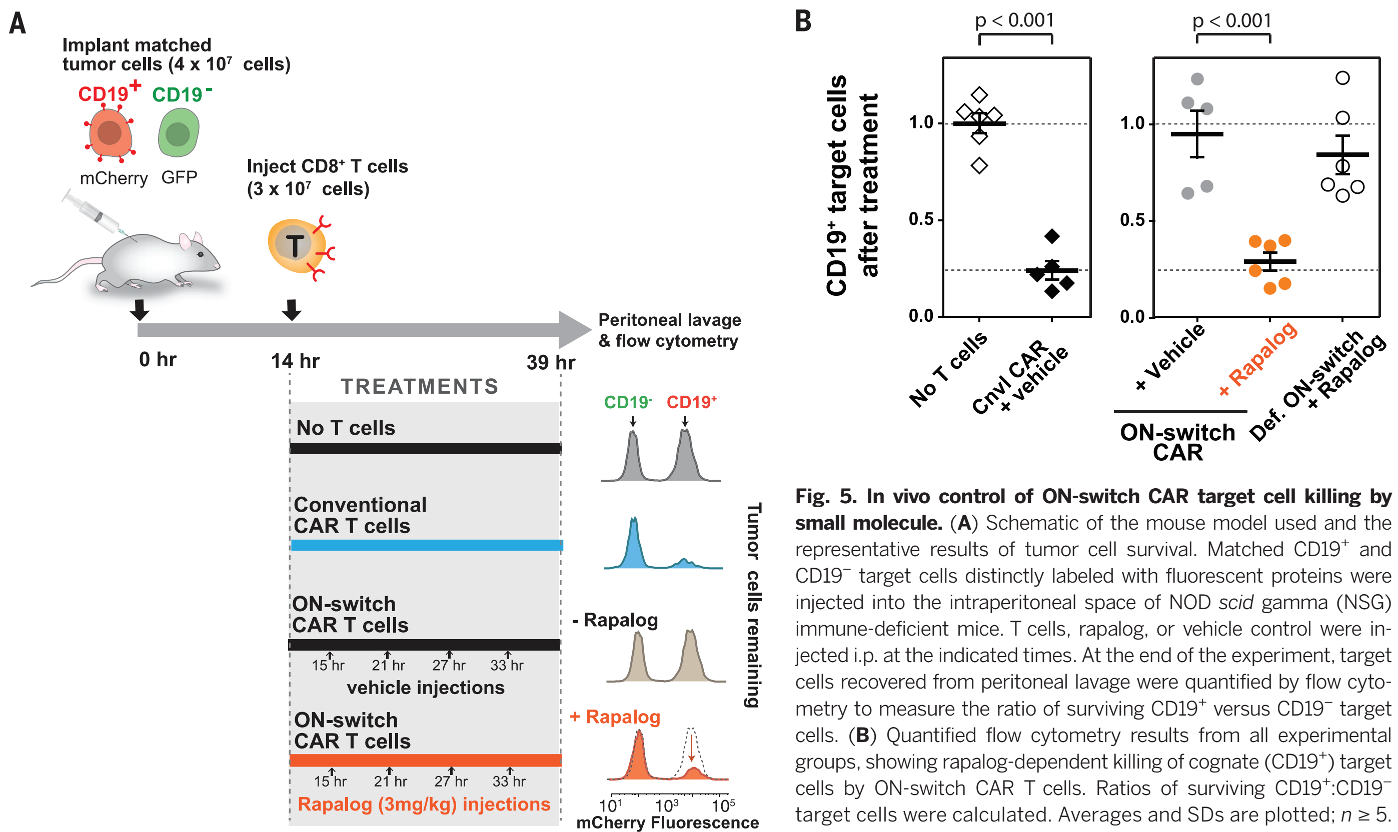


Fig. 5. In vivo control of ON-switch CAR target cell killing by small molecule. (**A**) Schematic of the mouse model used and the representative results of tumor cell survival. Matched CD19$^+$ and CD19$^-$ target cells distinctly labeled with fluorescent proteins were injected into the intraperitoneal space of NOD *scid* gamma (NSG) immune-deficient mice. T cells, rapalog, or vehicle control were injected i.p. at the indicated times. At the end of the experiment, target cells recovered from peritoneal lavage were quantified by flow cytometry to measure the ratio of surviving CD19$^+$ versus CD19$^-$ target cells. (**B**) Quantified flow cytometry results from all experimental groups, showing rapalog-dependent killing of cognate (CD19$^+$) target cells by ON-switch CAR T cells. Ratios of surviving CD19$^+$:CD19$^-$ target cells were calculated. Averages and SDs are plotted; $n \geq 5$. *P* values to compare pairs of experimental groups were calculated with Student's *t* test.

39 hours) for collection of peritoneal lavage (44), from which recovered cells were analyzed by flow cytometry.

Relative to mice that did not receive any T cells, mice injected with conventional CAR T cells showed selective depletion of the CD19$^+$ K562 cell population (Fig. 5A). ON-switch CAR T cells produced a similar result, but only in mice treated with rapalog. Normalized numbers of remaining CD19$^+$ target cells (relative to numbers of remaining CD19$^-$ target cells) are plotted in Fig. 5B. These data show that the ON-switch CAR T cells, in the absence of rapalog, led to the same outcome as in conditions where no T cells were given or defective ON-switch T cells and rapalog were given. However, when the mice injected with ON-switch CAR T cells were also treated with rapalog, the decrease in CD19$^+$ cells matched that observed with the conventional CAR T cells.

These studies confirmed that the ON-switch CAR T cells can be effectively controlled with a small molecule in vivo. Both in vitro and in vivo, the engineered cells demonstrate no constitutive killing of target cells, but show selective killing of cognate target cells when exposed to the dimerization-inducing molecule. The rapalog we used in vivo is not ideal given its short half-life in plasma. However, we demonstrated that unrelated dimerization systems can be used to control the ON-switch CAR architecture (Fig. 2B). Thus, it is likely that heterodimerization systems optimized for in vivo pharmacokinetic properties and safety could be used to tightly control ON-switch CAR T cell activity in patients.

Safer therapeutic immune cells by integrating autonomous and user control

We engineered a class of synthetic T cell receptors that allow for effective exogenous control over T cell antitumor activity, including cytokine production, proliferation, and cytotoxicity. This receptor design is modular, in that customizable small-molecule dimerization systems can be used to gate signaling. As with conventional CARs, various extracellular domains for recognition of distinct ligand antigens can be used. Further analysis shows that the ON-switch design is also compatible with further customization through a mutated 4-1BB signaling domain in part I or a monomeric form of part II, if it is preferred that these individual components not associate with the endogenous TRAF or DAP10 molecules (figs. S10 and S11) (29, 45, 46).

The ON-switch receptor system depends on two combined inputs to trigger T cell activation: a disease-specific ligand and a small-molecule drug. This type of antigen ligand + small-molecule combinatorial control made possible by the ON-switch design might make adoptive T cell therapy safer, as the activities of an infused T cell population could be selectively regulated in a temporal and titratable manner to minimize both off-target and on-target toxicities (7). T cells engineered with an anti-HER2 CAR to treat metastatic colon cancer have caused severe and rapid cross-reaction with normal cells expressing low amounts of HER2 in the lung; CAR T cells first concentrate and transit through the lung immediately after infusion (16). This "first pass" toxicity involving lung, heart, and liver, which occurs immediately after T cell infusion, could potentially be limited by delaying activation of the T cells until after they have distributed throughout the body. Using locally administered small molecules, ON-switch CAR T cells could allow for titratable control over T cell activity, as well as the location of therapeutic action when combined with technologies that can locally deliver small molecules into targeted tissues (20).

Need to develop modules for orthogonal chemical control of engineered cells

More generally, this and related work highlights the value of orthogonal chemical control as an interface for any kind of cellular therapeutic agent. Toxicity concerns of cell-based therapies primarily stem from a lack of efficient methods to specifically communicate with and regulate the cells once they are in the patient (aside from systemic treatments such as immunosuppression). A small-molecule drug–inducible "suicide" switch that induces apoptosis in engineered T cells can be used to abort cellular therapeutics (18, 24). A modular, RNA-based system has also been

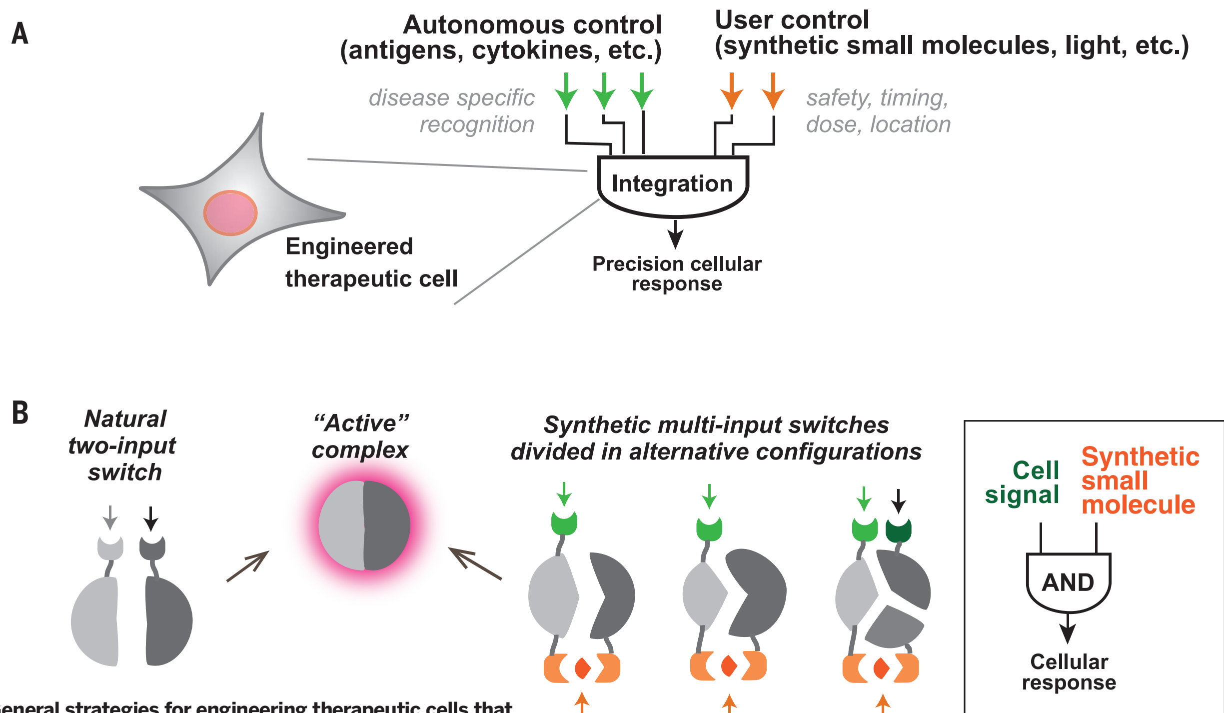


Fig. 6. General strategies for engineering therapeutic cells that integrate autonomous and user control. (**A**) Ideal therapeutic cells are expected to (i) produce potent therapeutic effects upon recognizing disease-specific signals and (ii) act in a temporally and spatially regulated manner. As illustrated in this work, cell-autonomous signaling in response to disease-specific inputs can be integrated with exogenous, user-supplied inputs to produce more precisely regulated therapeutic responses. (**B**) Regulated assembly into conditionally active complexes is commonly observed in natural regulatory systems. This strategy can be exploited to generate synthetic multi-input control by generating alternative split configurations of the active state that are conditionally assembled only with the proper combination of input molecules.

developed for small molecule–dependent cytokine production and proliferation of engineered T cells in vivo (*47*). Orthogonal ligands of G protein–coupled receptors can be used to guide T cell migration in vivo (*48*). The ON-switch CAR described in this study can be implemented along with these other synthetic control devices to produce "smart T cells" whose key therapeutic behaviors are individually under exogenous control.

This work also emphasizes the need for the development of additional orthogonal channels for molecular control. Reagents such as the rapalog molecule used here were primarily developed as tools for chemical biology studies, and they do not have ideal pharmacokinetic properties for clinical use. Advancement in cellular therapeutics may thus require the development of new classes of controller drugs that are optimized for clinical use in combination with engineered therapeutic cells. Such drugs should be safe and bio-inert, have good pharmacokinetic properties, and have cognate response modules that can be flexibly incorporated into the molecular machinery of the engineered cells (*48*–*50*). Other modalities of control—such as light, which can be detected by optogenetic modules (*51*–*55*) or other physical signals—could in principle also be useful as additional channels to control therapeutic cells. Combining the tools and strategies of chemical biology with genetic engineering may produce more controllable cellular therapeutic agents with improved therapeutic profiles.

Strategies for engineering new layers of combinatorial control in synthetic receptors

The type of cellular control engineered here represents the general principle of integrating autonomous control (e.g., targeting of disease ligands) with user control (e.g., small molecules) (Fig. 6A). As we begin to engineer more cellular therapies, integration of these two modes of cellular regulation is likely to become increasingly important. Regulation by user inputs allows the physician more precise control over the timing, dose, and location of a cellular action and thus more flexible safety control.

This work also demonstrates that it is possible to engineer additional layers of positive control into an already complex synthetic receptor. The engineering of the CAR performed here is analogous to changes in regulatory function that occur in signaling proteins over the course of evolution, when new regulatory inputs are layered and integrated with one another. We used the simple strategy of harnessing controlled molecular complex assembly as a way to achieve combinatorial multi-input regulation (Fig. 6B). The active signaling complex at the T cell plasma membrane is normally conditionally assembled when the TCR and co-receptors are stimulated, whereas the CAR preassembles this complex. Thus, to achieve multi-input control, we have resplit this assembly in a different configuration and in a manner such that its reassembly is dependent on small-molecule binding. This technique of synthetic division and reassembly constitutes a robust strategy for integrating and layering different control modules over cellular responses in order to develop synthetic combinatorial control systems that mimic the precision of natural cellular signaling responses (*56*, *57*).

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SUPPLEMENTARY MATERIALS

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