Inducible Gene Switches with Memory in Human T Cells for Cellular Immunotherapy

Deboki Chakravarti,[†,‡] Leidy D. Caraballo,[†,‡] Benjamin H. Weinberg,[†,‡] and Wilson W. Wong*[,†,‡]

†Department of Biomedical Engineering, Boston University, Boston, Massachusetts 02215, United States
‡Biological Design Center, Boston University, Boston, Massachusetts 02215, United States

S Supporting Information

ABSTRACT: Cell-based therapies that employ engineered T cells—including those modified to express chimeric antigen receptors (CARs)—to target cancer cells have demonstrated promising responses in clinical trials. However, engineered T cell responses must be regulated to prevent severe side effects such as cytokine storms and off-target responses. Here we present a class of recombinase-based gene circuits that will enable inducible, one-time state switching in adoptive T cell therapy using an FDA-approved drug, creating a generalizable platform that can be used to control when and how strongly a

gene is expressed. These circuits exhibit memory such that induced T cells will maintain any changes made even when the drug inducer is removed. This memory feature avoids prolonged drug inducer exposure, thus reducing the complexity and potential side effect associated with the drug inducer. We have utilized these circuits to control the expression of an anti-Her2-CAR, demonstrating the ability of these circuits to regulate CAR expression and T cell activity. We envision this platform can be extended to regulate other genes involved in T cell behavior for applications in various adoptive T cell therapies.

KEYWORDS: CAR, immunotherapy, synthetic biology, genetic circuits, recombinase
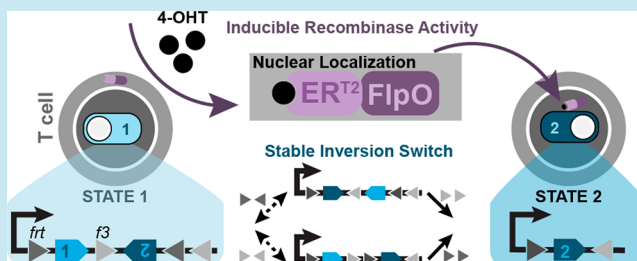
T cells have emerged as a promising candidate for cell-based therapies, and engineering of T cells to express antigen-specific chimeric antigen receptors (CARs) has enabled programmable targeting of cancer cells.[1−4] Multiple clinical trials with CARs against B cell cancers have driven up to a 90% complete response rate in patients,[5−8] and several CAR T cell products targeting acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma have been approved for clinical use in the United States.

Despite these promising clinical results, there are significant safety and efficacy considerations with CAR T cell therapies, often mirroring fundamental regulatory challenges of the immune system.[9,10] For example, the immune system naturally seeks to prevent autoimmune reactions by selecting against highly autoreactive T cells during their development in the thymus.[11] However, engineered cancer-specific receptors often target markers that, while overexpressed on tumor cells, may still be found at lower levels in healthy tissues.[12] These modified cells have the potential for an autoreactive "on-target, off-tumor" response, which has been observed and proven fatal in at least one clinical trial.[13] In addition, there are numerous regulatory checks that prevent the immune system from responding too strongly against pathogens and causing systemic harm, checks that may become disrupted by engineered T cells. CARs, in particular, can instigate a strong cytokine release in response to antigen stimulation, accelerating the immune response to potentially fatal levels. This cytokine release syndrome (CRS) has been observed in several

CAR T cell clinical trials,[7,8] and a regimen of immunosuppressive drugs is often required to ameliorate the response.[5]

These safety considerations point to the dangerous aspects of what is otherwise the major advantage of cell-based therapies: the ability to drive strong responses based on the cell's own machinery. Targeted cytotoxicity is fundamental to the power of T cell therapies, but it is also the basis of its potential harms. This challenge is compounded by the immense cost—both in time and money—of cell-based therapies, which makes it difficult to iterate this therapy over and over again until it meets a patient's individual needs.

T cell therapies would thus greatly benefit from the development of tools that allow greater control over cell behavior, enabling an already personalized therapy to be customizable toward a patient's immediate and changing needs. Advances in genetic engineering and synthetic biology have provided significant insight into both the design and implementation of such controls in the form of synthetic receptors, protein-based switches, genetic circuits, and genome editing tools.[9] These tools have in turn been used to create "ON-Switch" CARs,[14] combinatorial activation systems,[15,16] doxycycline-inducible CARs,[17] antibody-inducible CARs,[18] kill switches,[19−21] pause switches,[22] tunable receptor systems,[23−25] proliferation switches,[26] and a universal "off the shelf" T cell.[27] These systems all reflect the tremendous potential of synthetic

biology approaches in developing safer and more powerful forms of T cell therapy that can be customized to fit each patient.

While these technologies offer important forms of control over T cell behavior, their designs are accompanied by limitations. For example, while kill switches provide vital control in cases where the T cells are toxic to the patient, completely shutting off the therapy may be an extreme response for patients who only require a slight modification of the therapy to abrogate negative reactions. Some approaches are also only limited to certain types of therapy, such as the many CAR receptor designs that strictly provide added flexibility and control to CAR T cell therapy. In addition, certain inducible systems like the "ON Switch" CAR require the drug-inducer to be continuously provided to maintain the ON state, which may be less ideal if permanent changes are required for a patient. Furthermore, prolonged drug-inducer exposure may be detrimental to patients if the inducer has a less than ideal safety profile, even when the drug is FDA-approved. While these approaches will advance the scope of potential T cell therapies, developing further technologies that are compatible with them may help to expand their use.

To address the need for advanced control of T cell responses for various immunotherapy applications, we have developed a drug-inducible genetic circuit platform that acts as a one-time state switch in human primary T cells. This platform is designed to be lentivirus-compatible, which will facilitate genetic modification of human primary T cells. Our system also has memory capability that reduces the need for prolonged drug administration to maintain gene expression level. We have adapted these circuits to control CAR expression, creating a variety of circuits, including an On Switch (ON), Off Switch (OFF), and Expression Level Switch (EXP) that controls CAR expression, alters T cell behavior, retains memory, and exhibits activity that can be tuned *via* drug dosage and duration. The ON and the OFF switch enable control over when a CAR is expressed in a cell, while the EXP switch provides a novel mechanism to combine the memory capacity of the circuit with the ability to modulate the level of CAR expression within each cell. All three forms of control provide paths toward more complex therapeutic strategies, and these gene switches represent the most versatile switches in T cells and have the potential to improve the safety and efficacy of T cell immunotherapy.

■ RESULTS

Recombinase-Based Gene Switch for Controlling CAR Expression. To implement a lentivirus-compatible, two-state switch with memory in T cells, we have adapted the recombinase-based flip-excision (FLEx) stable inversion switch for T cells. Recombinases are enzymes that can perform inversion or excision steps on DNA based on the relative orientation of DNA recognition sites. Recombinases were chosen for this work because they have demonstrated exceptional versatility and performance for engineering of gene regulation systems in mammalian cells.[28] The FLEx switch was initially designed using the Cre/*lox* system to regulate gene expression in mammalian cells *via* retroviral transduction of the switch.[29] This system relies upon the availability of orthogonal *lox* variant sites that are recognized by the Cre recombinase but do not interact with other variant sequences. Activation of the FLEx switch with recombinase begins with an unstable inversion step followed by a stable

excision step, effectively removing one sequence of DNA and inverting another (Figure 1). Due to the configuration and of
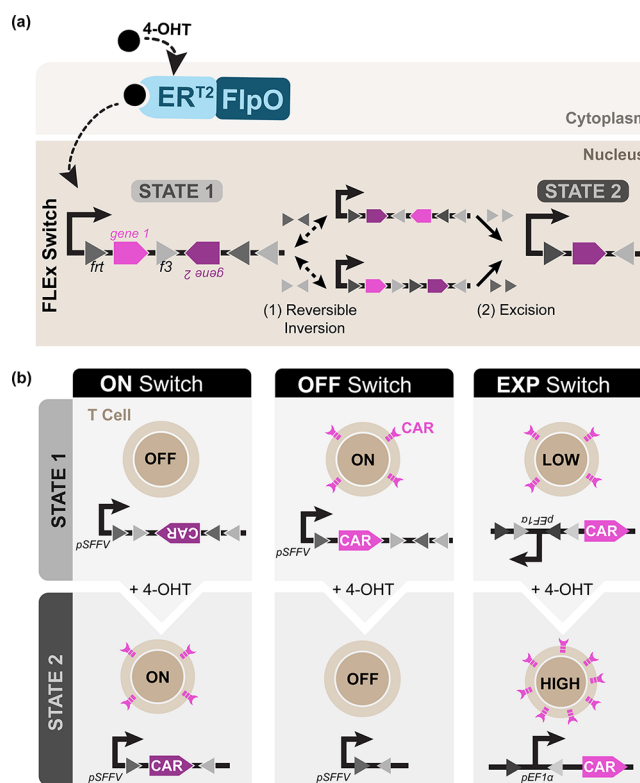

Figure 1. FlpO recombinase based FLEx switch design. (a) Mechanism of the 4-OHT-inducible FLEx switch using FlpOER^{T2}. Binding of 4-OHT to the ER^{T2} domain drives nuclear localization of the FlpO recombinase, initiating a reversible inversion upon either the *frt* or *f3* recognition site and then an irreversible excision upon the remaining site. By encoding sequences representing State 1 and State 2 between the recognition sites, induction of FlpO activity stably shifts the cell from State 1 to State 2. (b) Design of the ON, OFF, and the Expression (EXP) level switch to control expression of CAR. The ON and OFF Switch express the CAR gene under State 1 and State 2 respectively. The EXP switch alters the orientation of the EF1α promoter relative to a CAR gene to take the cell from low CAR expression to high expression.

recombination sites in the final product, this stable inversion switch can only be performed one time. The overall product is a one-time state switch that—when genes are encoded between the recombination sites—can stably alter gene expression *via* recombinase activity.

The FLEx switch exhibits several features that make it both applicable and beneficial toward T cell therapies. The stable inversion capability means that unlike a transcriptionally inducible gene system, this circuit contains memory: when recombinase activity is terminated, changes made to the cells are maintained. This property is ideal for therapeutic strategies that seek a permanent change to T cell behavior without requiring continuous drug intake. It also enables changes to remain robust in response to rapid changes in proliferation that may dilute protein levels. In addition, the FLEx switch avoids the use of genetic elements that cannot be implemented with viruses. For example, transcription termination sites are a powerful and simple element that enables the design of complex recombinase-based logic systems in mammalian cells.[28] However, transcription termination sites interfere
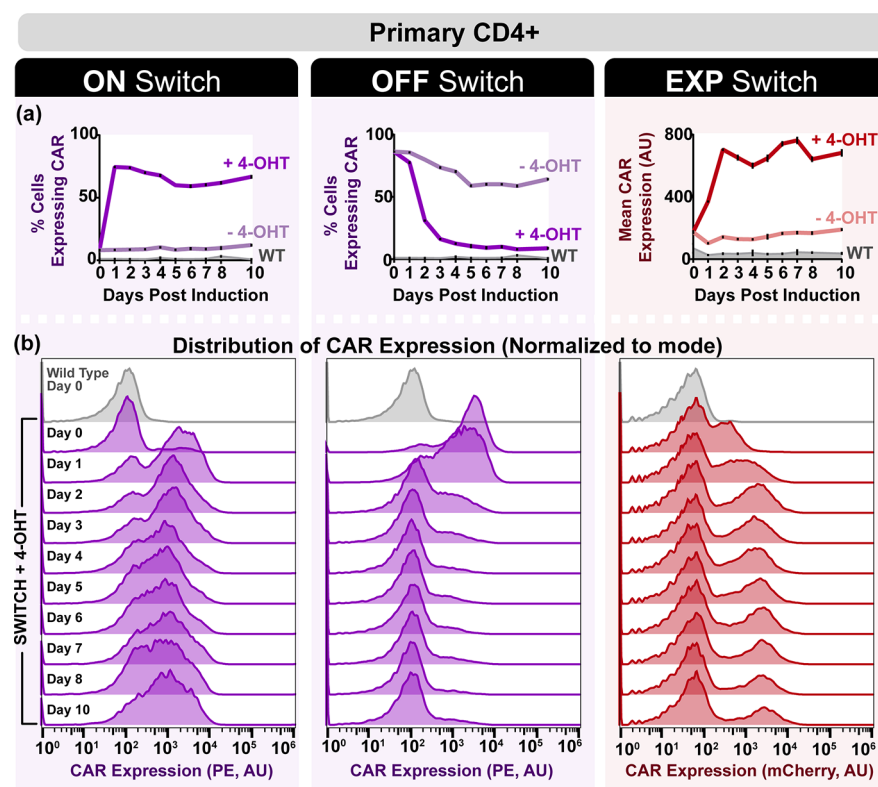
Figure 2. FlpO can be used to create ON, OFF, and Expression level switches to control αHer2-CAR expression in human primary CD4+ T cells. (a) Time course data for recombinase-positive cells with or without drug addition (1 μM 4-OHT). Samples were obtained in triplicate from each induced or noninduced culture and then plotted as mean and standard deviation. The ON and OFF switches are presented as percent cell expressing the αHer2-B1D2-CAR. The EXP switch is presented as the mean αHer2-G98-CAR expression level in arbitrary units (AU). For all circuits, CAR expression in +4-OHT cells was significantly different from −4-OHT cells starting 1 day postinduction (unpaired two-tailed *t* test with Holm−Sidak adjustment, *p* < 0.0001). (b) Change in distribution of CAR expression level (AU) in recombinase-positive cells days following 4-OHT induction.

with the reverse transcription process of viral integration, and sequences containing transcription termination sites cannot be integrated into the T cell genome *via* viral transduction, rendering them unusable in many clinical settings that rely upon viral transduction for engineered T cell production. The FLEx switch does not contain transcriptional stop sites or other elements that would interfere with viral integration, and it has demonstrated lentiviral compatibility.[30]

While the FLEx switch has been designed with the Cre/*lox* system, Cre exhibits toxicity[31,32] in mammalian cells due to the presence of pseudo-*loxP* sites in the genome. This genotoxicity requires careful tuning to be mitigated.[33] We initially developed a Cre/*lox*-based FLEx switch into Jurkat T cells and observed high toxicity upon Cre induction (Supplementary Figure S1). We adapted the FLEx switch to operate with the FlpO/*frt* recombinase system instead, as the Flp recombinase has not been reported to be toxic.[34,35] Parallel to Cre recognition of the *lox* DNA recognition sites and variants upon the *lox* sequence, the FlpO recombinase recognizes and acts upon *frt* recognition sequences and variants including the *f3* sequence, which we used with the *frt* sequence to construct a FlpO-based FLEx switch. A similar FlpO-based FLEx switch has been designed using other variant *frt* sites.[36] To control recombination, we used FlpO conjugated to the mutated estrogen receptor ER[T2],[37] which localizes the recombinase to the cytoplasm.[29,38] When the ER[T2] domain binds to 4-hydroxytamoxifen (4-OHT), a metabolite of the drug tamoxifen, FlpOER[T2] is localized to

the nucleus where the recombinase can act upon the FLEx switch (Figure 1a). We chose the tamoxifen-inducible FlpO for our circuit design because tamoxifen is an FDA-approved drug, which will facilitate implementation into the clinic. We have adapted the FlpOER[T2]/*frt* FLEx switch design to alter the orientation of CAR genes to create a stable ON and stable OFF switch (Figure 1b). In addition, we have designed an "Expression Level" (EXP) switch that takes advantage of the unbalanced bidirectional activity of the human EF1α promoter, controlling the orientation of promoter relative to the CAR gene to stably alter cells from low expression to high expression of CAR (Figure 1b).

Induction of Recombinase Activity Drives Changes in CAR Expression. We transduced human primary CD4+ T cells with two lentiviruses: one virus that contained a constitutively expressed FlpOER[T2], and another expressing either the ON, OFF, or EXP switch controlling the expression of an αHer2-CAR. We sorted cells for FlpOER[T2] expression and then induced with 4-OHT, a metabolite of tamoxifen, and observed changes in CAR expression *via* flow cytometry. All switches contained a CAR expressing an extracellular myc epitope tag that could be detected *via* antibody staining, but CAR expression in the EXP Switch was measured through an mCherry fluorescent tag directly conjugated to the CAR. However, to reduce the potential of altered CAR degradation rates due to the mCherry fluorescent tag, ON and OFF Switch cells expressed a CAR that lacked the fluorescent tag, and

instead CAR expression in these cells was measured by antibody staining for the myc epitope.

All three circuits exhibited significant changes in anti-Her2 CAR expression in recombinase-positive CD4+ T cells within 1 day of induction (Figure 2). The ON switch, in particular, demonstrated fast kinetics, reaching its maximal percentage of switched cells within 1 day. Meanwhile, OFF Switch cells demonstrated a loss in CAR+ cells within 1 day of induction, but the population required 6 days to stabilize (<10% cells expressing CAR). The slower dynamics of the OFF Switch compared to the ON Switch is likely due to the need to degrade and dilute CAR expression, making the OFF switch more reliant upon both growth and protein degradation rates.

The EXP Switch exhibits an increase in mean CAR expression across all recombinase-positive cells, though only approximately 20% of the cells express CAR. Indeed, all three circuits demonstrate that our populations are not homogeneously expressing all components of the circuit. In addition to variations in recombinase and CAR expression (Supplementary Figure S2), not all induced ON Switch cells express CAR (Figure 2). Nor do all uninduced OFF and EXP switch cells express CAR (Figure 2). This population heterogeneity is likely due to transduction inefficiency, which appears to have the greatest effect in implementing the EXP switch as apparent in its bimodal distribution throughout induction, and could additionally be affected by differences in viral integration location between cells. Indeed, when we integrated these circuits into Jurkat T cells, which generally exhibit greater ease of transduction, we observed cell populations that—with or without drug—exhibited greater homogeneity in all three switches (Supplementary Figure S3).

Two key markers of circuit performance are basal switching and switching completeness. A small population (∼8%) of ON Switch cells express CAR prior to induction, which indicates a low level basal FlpOER^{T2} activity (Figure 2). This basal level of CAR expression appears to be connected to a higher level of recombinase expression (Supplementary Figure S2). Switching completeness is better observed in the OFF switch, where less than 10% of cells still express CAR at the end of induction. In addition to these markers of circuit activity, we observed high viability and mostly comparable cell growth between uninduced and induced cells over the course of induction (Supplementary Figure S4), suggesting that toxicity of FlpO recombinase is minimal in T cells.

Changes in CAR Expression Impact T Cell Responses to Target Antigen. The induced changes in CAR expression by our switches are expected to alter T cell responses to antigen stimulation. We continued with switches that controlled expression of an αHer2-CAR and quantified the activation of circuit-expressing Jurkat T cells by stimulating them with plate-bound Her2 antigen and measuring the expression of an integrated NFAT (nuclear factor of activated T cells)-GFP transcription reporter, which produces GFP in response to binding of the NFAT transcription factor that is produced during T cell activation[39] (Figure 3). We observed that induced ON switch T cells, which express a CAR after 4-OHT-induction, can be activated with Her2. There was a low level of NFAT reporter expression at higher Her2 doses that suggests some basal CAR expression in uninduced cells, which could be due to a low level of basal recombinase activity. With the OFF switch, we observed that 4-OHT-induced cells have low reporter activity when stimulated with Her2, corresponding to the loss of CAR expression. The remaining level of
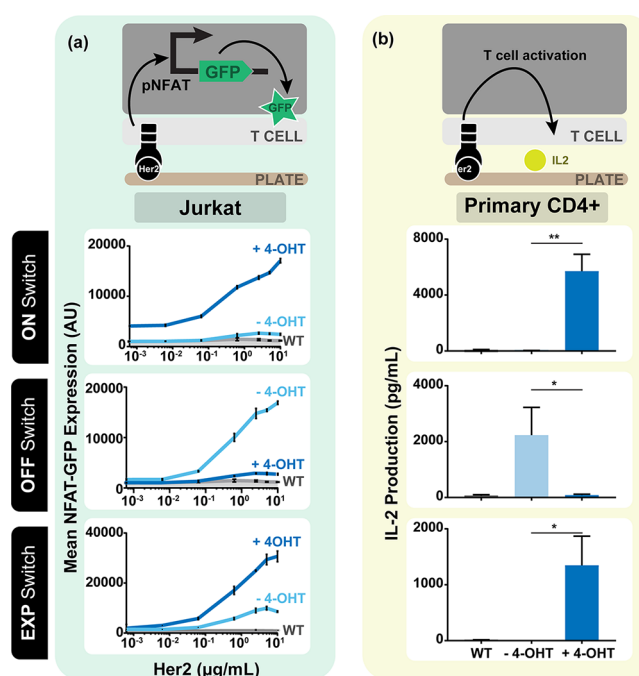

Figure 3. αHer2 CAR activation in T cells containing the recombinase switches. Switch-expressing Jurkat T cells (a) and human primary CD4+ T cells (b) for control of expression of an αHer2-G98-CAR were exposed to 1 μM 4-OHT prior to CAR activation for 5 days (Jurkat, ON and OFF), 8 days (Jurkat, EXP), and 9 days (Primary CD4+, all). Jurkat cells were exposed to different concentrations of plate-bound Her2 protein, and primary T cells were exposed to 5 μg/mL Her2. An NFAT-GFP transcription reporter and IL-2 were measured for Jurkat and primary T cell, respectively. Cells were plated against Her2 antigen in triplicate, and both NFAT-GFP (arbitrary units, AU) and IL-2 (pg/mL) were plotted as mean and standard deviation. NFAT-GFP expression was significantly different between +4-OHT and −4-OHT Jurkat T cells for all three circuits at all Her2 dosages (unpaired, two-tail t test with Holm−Sidak adjustment, $p < 0.01$) with the exception of the EXP switch exposed to the lowest Her2 dosage (0.0625 μg/mL). Statistical significance in IL-2 production was determined by unpaired, two-tail t test (*$p < 0.05$, **$p < 0.01$). Activation of primary T cell IL-2 production was repeated on consecutive days (at 1 μg/mL on day 8 and 5 μg/mL on day 9 for EXP switch, at 5 μg/mL on days 7−9 for ON and OFF switch), and day 9 is reported here.

NFAT activity in induced cells may be due to incomplete FLEx circuit switching, which could result from factors such as inaccessibility to the integration site. These results are mirrored in ON and OFF switch CD4+ primary T cells that are activated 10 days after induction, where activation is measured *via* production of the IL-2 cytokine (Figure 3). For EXP switch-expressing Jurkat T cells, we observed that uninduced cells (which are in the low CAR expression state) were activated in the presence of Her2 at a level higher than the basal activation in uninduced ON switch cells (Figure 3). The increase in CAR expression after 4-OHT induction led to a corresponding increase in NFAT-GFP expression.

Interestingly, while we observed this effect for a low-affinity Her2-CAR (C6.5G98A, $K_D = 3.2 \times 10^{-7}$),[3] when the expression level switch is applied to Her2-CARs with increased affinity (C6.5, $K_D = 1.6 \times 10^{-8}$; C6MH3-B1, $K_D = 1.2 \times 10^{-10}$; C6-B1D2, $K_D = 1.5 \times 10^{-11}$),[3] the effect on NFAT-GFP activation levels is reduced at high levels of Her2 antigen, illustrating that the level of T cell activation is dually
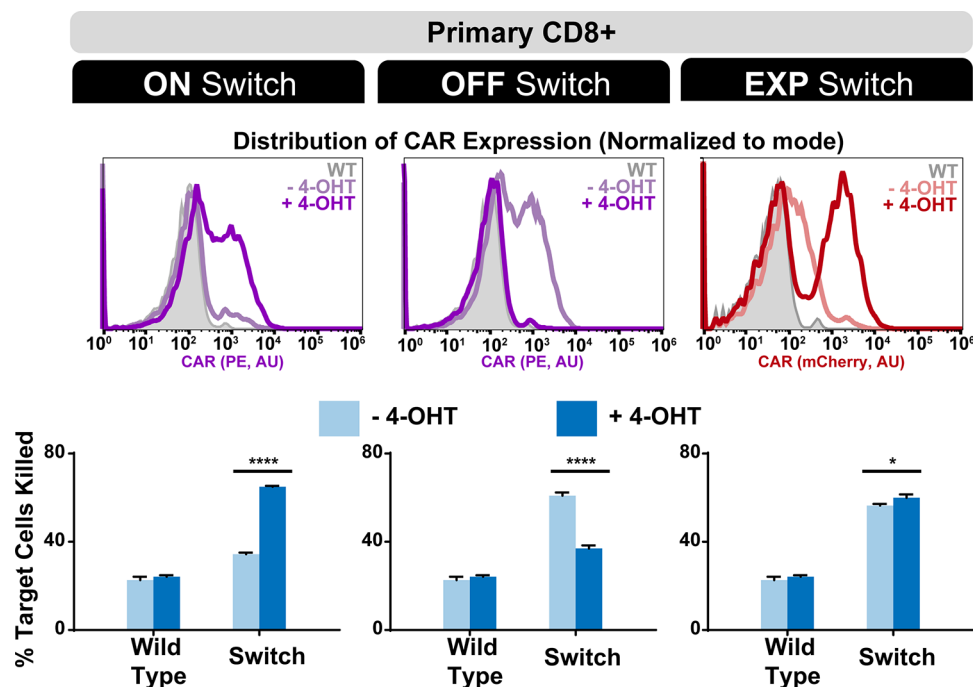
Figure 4. Modulation of cell-killing activity of T cells containing the recombinase switches. Human primary CD8+ T cells containing the ON, OFF, or EXP switch controlling expression of an αHer2-G98-CAR were exposed to 1 μM 4-OHT prior to CAR activation for 6 days and then plated with Her2+/GFP+ NALM6 cells at an effector:target ratio of 1:2 for 20 h. (Top Panel) Histogram of CAR expression in induced and uninduced CD8+ T cells 7 days postinduction. (Bottom Panel) Percentage of target NALM6 cells killed by primary CD8+ T cells. Cells were plated in triplicate, and % target cells killed was plotted as mean and standard deviation. Statistical significance in cell killing was determined by unpaired, two-tail t test (*p < 0.05, ****p < 0.0001). Cell killing assay performed three consecutive days (5−7 days postinduction), and day 6 cell killing is reported here.
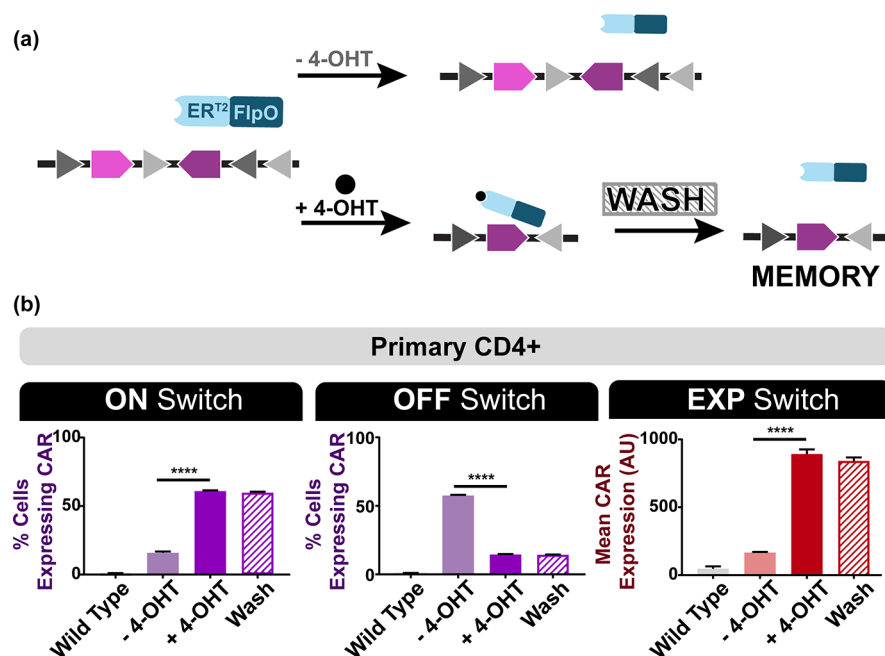

Figure 5. Recombinase switches in primary T cells maintained CAR expression memory after the removal of the inducer. (a) Experimental work flow characterizing the switch circuit memory. (b) CAR expression from the ON, OFF, or EXP switch with or without washing the 1 μM 4-OHT after 2 days of 4-OHT exposure. The ON and OFF switches are presented as percent cell expressing the αHer2-B1D2-CAR. The EXP switch is presented as the mean αHer2-G98-CAR expression level in arbitrary units (AU). Samples were obtained in triplicate from each induced or noninduced culture and then plotted as mean and standard deviation. Statistical significance in CAR expression was determined by unpaired, two-tail t test (****p < 0.0001).

modulated by the CAR expression level and its antigen-scFv affinity (Supplementary Figure S5), corroborating similar observations by others.[3,16,40,41] Primary CD4+ T cells

expressing the EXP switch also demonstrate an increase in IL-2 production when activated against Her2. Expression of IL-2 in uninduced EXP cells was low and similar to wild-type,
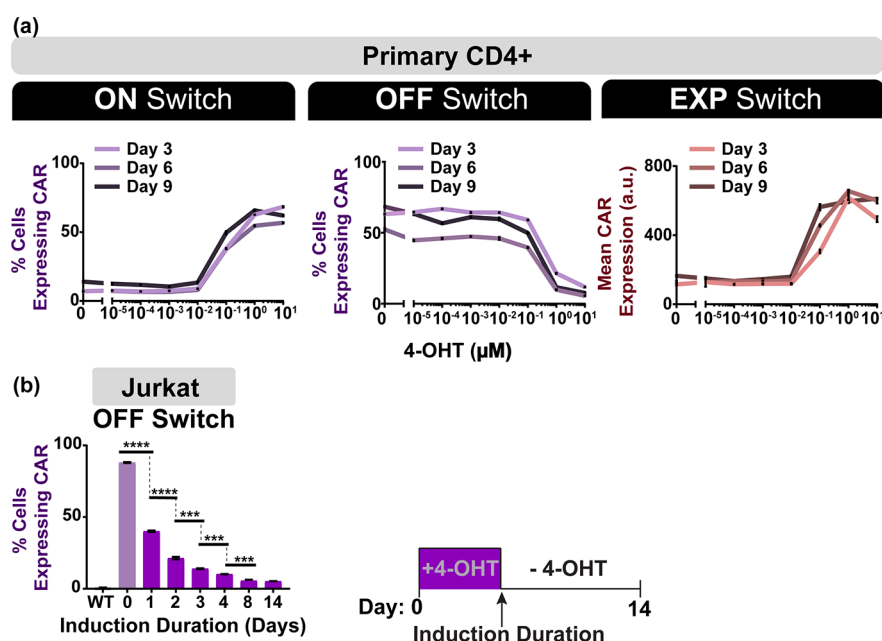
Figure 6. Dose and duration of 4-OHT induction govern the CAR expression from the recombinase switches. (a) CAR expression as a function of 4-OHT concentration for the ON, OFF, and EXP switch. The dose response was performed once and measured at 3 points following induction. Different lines represent the CAR expression at 3, 6, or 9 days after 4-OHT exposure. The ON and OFF switches are presented as percent cell expressing the αHer2-B1D2-CAR. The EXP switch is presented as the mean αHer2-G98-CAR expression level in arbitrary units (AU). (b) CAR expression as a function of 4-OHT exposure duration. Cells were washed at the indicated day after 4-OHT exposure, and αHer2-C65-CAR expression was measured 14 days after the initial drug addition. Samples in (a) and (b) were obtained in triplicate from each induced or noninduced culture and then plotted as mean and standard deviation. Statistical significance in cells induced for n days compared to cells induced for $n-1$ days was determined by unpaired, two-tail t test with Holm−Sidak adjustment (***$p < 0.001$, ****$p < 0.0001$).

which may be due to a combination of the low CAR expression within the cells, the small population of cells expressing CAR overall, and the low affinity of the CAR.

To confirm that these changes in T cell behavior could also be observed in primary T cells, we induced switch-expressing primary CD8+ T cells for 6 days and then plated the cells with an engineered Her2+/GFP+ NALM6 cell line overnight. We measured GFP expression in the sample *via* flow cytometry to ascertain the fraction of target NALM6 cells that were killed (Figure 4). The ability of the CD8+ T cells to kill these target NALM6 cells was altered by the addition 4-OHT: induced ON and EXP switch cells demonstrated an increase in killing, and induced OFF switch cells demonstrated a loss in killing compared to their uninduced counterparts. These results both confirm that the ON, OFF, and EXP circuits exhibit switching behavior in primary CD8+ T cells, and that these changes in CAR expression affect T cell behavior. Similar to the basal NFAT-GFP activation in ON switch Jurkat T cells, these results do indicate some basal killing in uninduced ON switch cells, as well as some remaining killing capability in the induced OFF switch cells—behaviors that could require further tuning for practical implementation. However, much of the basal killing is derived from the CD8 T cells alone, even without a CAR.

Switch Cells Exhibit Memory When Drug Is Removed. One unique feature of the recombinase-based switch that is not found in other existing gene switches is memory: removal of the drug will maintain any changes made to the cell. Cells that have been switched on will stay on, cells that have been switched off will stay off, and cells that now express greater CAR will continue to express greater CAR. To test this feature in our cells, we compared CAR expression 15 days post-

induction in recombinase-positive primary CD4+ T cells that had been induced for only 2 days *versus* cells that had been continuously induced for the entire 15 day period. For each switch type, the level of CAR expression for cells that had been continuously induced was the same as the level of CAR expression for cells that had their induction stopped after 2 days, indicating each switch type was able to maintain memory of the changes made to the cell even after induction had ended (Figure 5).

Tunability of Switching Can Be Driven by Drug Dosage and Duration. While these circuits ostensibly provide a two-state system, the percentage of cells that switched states may be tunable by applying the inducer at different concentrations. Therefore, we treated recombinase-positive primary CD4+ T cells with a range of 4-OHT dosages and showed that the percentage of cell population switched states in the ON and OFF switch can indeed be modulated (Figure 6a). In addition, the level of mean CAR expression in the EXP switch can be further tuned *via* drug dosage. The trend in CAR expression *versus* drug dosage remains consistent when measured 3, 6, and 9 days postinduction for all three switches. However, there is a decrease in CAR expression on day 6 for both low dosage OFF switch and high dosage ON switch that is likely the result of day-to-day fluctuations in the flow cytometer.

In cases where the switch takes longer to work, the memory feature enables further tuning of switch dynamics. In particular, varying the duration of drug dosage resulted in varying degrees of switching in Jurkat OFF Switch cells (Figure 6b, Supplementary Figure S6). However, these results did not extend to primary T cells, where all switches expressed the same level of CAR when induced for only 2 days compared to

cells that were induced for much longer (Figure 5). Jurkat ON and EXP switch cells also did not exhibit drug duration tunability (Supplementary Figure S6).

■ DISCUSSION

We have presented a recombinase-based genetic circuit system that allows for increased control of primary T cell behavior. The circuits we have presented here are a lentiviral-compatible system with memory capability such that continual addition of the drug is not required for maintenance of any desired changes to the cellular state. This capacity for memory would be particularly important for therapeutic strategies that require permanent changes to be made to the cell, such as permanently shutting off expression of a particular gene, enabling only a temporary drug intake to make changes in lieu of asking a patient to continuously consume the drug. These switches can be used to turn a gene ON or OFF, as well as to stably alter the level of gene expression. In addition, we have demonstrated that the circuit's control of CAR expression extends to an effect on functional outputs like IL-2 production in CD4+ T cells and cell killing in CD8+ T cells.

The activity of these circuits provides a number of advantages for patients who require a tunable but permanent change made to the behavior of their engineered T cells. While transiently inducible systems, such as a the drug-dimerizable "ON Switch" CAR, can be powerful forms of control, the memory capacity of the circuits described here will enable the development of a broader class of therapeutic strategies that can make long-lasting changes to T cell behavior without requiring constant drug induction. Thus, this system provides a potential practical advantage, minimizing the duration of drug exposure, and potentially reducing the complexity of the treatment and possible side effects of the drug-inducer. While the ability of the OFF switch to reduce the number of CAR-expressing T cells is similar to the implementation of an inducible kill switch, our platform does not kill the T cell. The availability of this nonapoptotic method to reducing CAR expression can enable the continued use of the engineered cell with other T cell-controlling technologies—such as controls over proliferation or localization—and provide doctors with more tunable parameters to respond to an individual's needs.[9] Moreover, the inducible kill switch lacks the ability to serve in therapeutic strategies that require for CAR expression to be turned on in a controlled manner, such as in our ON switch, or to modulate gene expression levels similar to our EXP switch, reflecting the flexibility in therapeutic design offered by our one-time state switch platform. Thus, our system confers a potential practical advantage.

In addition, the tunability of the circuit behavior enables not only the preprogrammed State 1 and State 2 expressed within the circuit, but also a range of CAR expression across the population that could further tune therapeutic activity within the patient. The potential of induction duration to tune circuit behavior may also expand the available therapeutic range. At a 4-OHT concentration of 1 μM, we were only able to observe this duration dosage property in OFF Switch Jurkat T cells. There are many potential factors driving the varying impact of drug duration on the circuits in different cell types. For example, variations in cell division rates between primary and Jurkat T cells may impact the rate of CAR dilution, which would in turn drive differences in OFF switch dynamics in the two cell lines that become magnified when drug is added for shorter durations. It is also possible that the basic mechanical

differences of creating more protein (as seen in the ON and EXP) switch compared to destroying protein (as in the OFF switch), which we see creates differences in the induction dynamics, may then impact the effect of drug duration (at least as observed in Jurkat T cells). While the results described here do not indicate successful drug duration tuning of circuits in primary T cells for the given dosage and durations, the results in Jurkat T cells suggest that there is a capacity for the memory capability of the FLEx switch to enable drug duration tuning. Further computational and experimental work exploring the combined parameter space of drug dosage (particularly lower drug concentrations) and drug duration, particularly *in vivo*, may reveal more tunable behavior for all three switches in primary T cells. Indeed, *in vivo* use of these switches will require greater study and investigation into the kinetics of both drug delivery and induction of cells.

Our genetic circuits also enable more complex T cell therapeutics by providing a platform to program two different therapeutic states into a cell simply by expressing a particular gene of choice within the switch cassette. This increased complexity will expand therapeutic strategies that can be implemented, as it will enable an easy change from one state of therapy to another based on a patient's individual needs. While we have focused on its use with CARs in this work, our circuit is also compatible with other forms of T cell therapies that could benefit from control of other genes.

Our use of FlpOER[T2] takes advantage of the power of drug-inducible recombinases to create powerful genetic technologies, as well as the improved viability of cells expressing FlpO over the commonly used Cre recombinase. However, one potential limitation of recombinases is their immunogenicity due to their nonhuman origins (FlpO, for example, is derived from yeast). To mitigate transgene immunogenicity, one strategy is to leverage genome editing tools to eliminate the gene B2M, an important component for antigen display through class I human leukocyte antigen (HLA).[42,43] Inhibiting the capacity for antigen display in engineered T cells reduces the potential to present epitopes derived from the components of our circuit, which in turn could act as a safeguard to prevent other immune cells from targeting these engineered cells. This strategy has also been combined with HLA-E overexpression to reduce immunogenicity against pluripotent stem cells by preventing the "missing-self" elimination driven by natural killer cells.[44] This approach would not only enable the development of "universal T cells" and provide a bank of "off-the-shelf" therapeutic T cells, it would enable the incorporation of genetic technologies comprised of proteins from diverse organisms. While the compatibility of these approaches with our platform and their combined viability in a therapeutic setting have yet to be established, with the rapid advancement in genome editing technologies, we are confident that transgene immunogenicity derived from FlpO can be addressed.

Optimized use of our recombinase-based switching will rely on careful consideration of the potential application. In particular, there are situations where low levels of basal activity or incomplete switching may carry enough risk that this approach will not be applicable, particularly if it results in low levels of cell killing that are still toxic to the patient. These issues could be further compounded by the selective proliferation of CAR-expressing T cells by antigen-presenting cells, which would further enhance the pool of either basal ON cells or incomplete OFF cells. Careful tuning of recombinase

expression or activity may provide further avenues to address these limitations, as could the incorporation of inducible kill switches to selectively kill cells that are not behaving as directed. Indeed, a version of this switch that controls not only CAR expression, but also a drug-inducible kill switch (which, similar to our OFF switch, is not 100% effective) could be an intriguing path to combine the power of these technologies. With this design, if a cell does not turn off expression of the CAR and the kill switch, we can induce the kill switch to kill off the remaining CAR/kill switch-expressing cells, leaving the "off" cells alive to perform other function that may have installed into them.

T cell therapies will require us not just to rely on the mechanics of the immune system, but also to understand the intricacies that are available and necessary for us to fine-tune in order to create a safe and effective treatment. With many developments and tools focused around developing one facet of control, having a platform of genetic circuits that can be applied in different ways creates a wider array of options available to implement T cell therapies.

■ METHODS

Circuit Construction. The circuit we described is comprised of two parts: the inducible recombinase and the FLEx switch. These components were cloned into separate lentiviral backbones using a combination of Gibson and traditional molecular cloning methods. The FlpOER^{T2} recombinase was cloned into the backbone followed by a T2A ribosomal skip sequence and an mTAG-BFP fluorescent marker.

The FLEx switch was designed using the *frt* and *f3* recombination sites. For the ON and OFF switches, the chimeric antigen receptor sequence was inserted between the recombination sites. The SFFV promoter was used to drive FLEx switch (and thus, CAR) expression. For the expression level switch, the EF1α promoter was inserted between the recombination sites such that the reverse promoter orientation was encoded in the 5′ to 3′ direction. The CAR was expressed downstream of the FLEx/reverse EF1α promoter.

Primary T Cell Isolation and Transduction. Blood was obtained from the Boston Children's Hospital, and primary CD4+ T cells were harvested using either the STEMCELL CD4+ enriched cocktail or STEMCELL CD8+ enriched cocktail in conjunction with the RosetteSep system. T cells were preserved at −80 °C in 90% FBS (Gibco) and 10% DMSO. T cells were maintained X-Vivo 15 media (Lonza) supplemented with 5% Human AB Serum (Valley Biomedical), 10 mM *N*-acetyl L-Cysteine (Sigma), and 55 μM 2-mercaptoethanol (Gibco). Through thawing and transduction, T cells were maintained with 100 units/mL recombinant IL-2 (Tecin, NCI BRB Preclinical Repository) and then 50 units/mL post-transduction.

Human embryonic kidney (HEK) 293 FT cells were transfected with lentiviral packaging plasmids and either the FLEx switch plasmid or the inducible recombinase plasmid in a T175 flask using polyethylenimine (PEI). One day after transfection, media was replaced with Ultraculture media (Lonza) supplemented with 100 U/mL Penicillin + 100 μg/mL Streptomycin (Corning), 2 mM L-Glutamine (Corning), 50 mM Sodium Butyrate (Alfa Aesar), and 1 mM Sodium Pyruvate (Lonza), and virus was collected three and 4 days after transfection by collecting and spinning the media, and retaining the supernatant. For CD4+ T cell transduction, virus

was concentrated through ultracentrifugation with 20% sucrose (Sigma) for 2 h at 4 °C and 22 000*g*. For CD8+ T cell transduction, virus was concentrated using PEG-8000: virus was mixed with a 40% (w/v) PEG-8000 and 1.2 M NaCl solution overnight and spun down for 1 h at 4 °C and 1600*g*.

T cells were thawed 2 days prior to transduction and activated with CD3/CD28 Dynabeads (Gibco) 1 day prior. Cells were transduced *via* spinfection: for CD4 + T cells, using half of concentrated virus, both inducible recombinase and switch viruses were spun onto the well of a 6-well retronectin (Clontech)-coated plates for 90 min at 1200*g*. The same protocol was followed for CD8+ T cells, but with the entire volume of concentrated virus. Activated primary T cells were then spun onto the virus plates for 60 min at 1200*g*.

Jurkat T Cell Maintenance and Transduction. Through transduction and general maintenance, Jurkat T cells were maintained in RPMI media (Lonza) supplemented with 5% fetal bovine serum (Gibco), 2 mM glutamine, and 100 U/mL penicillin+100 μg/mL streptomycin. Through analysis, cells were maintained in RPMI media supplemented with 10% fetal bovine serum and 2 mM glutamine.

Lentiviral transduction was used to produce T cell lines containing full circuitry (inducible recombinase and designated ON/OFF/EXP switch). HEK293FT cells were transfected *via* PEI in a 6 well plate with lentiviral packaging plasmids and the circuit component lentiviral plasmid to produce virus containing the specified component. Virus was collected 3 days after transfection.

Approximately 500 000 Jurkat NFAT cells—a line produced by the Weiss lab at UCSF[39] to express an NFAT-GFP activation reporter—were infected with 500 μL of the recombinase virus and 500 μL of the switch for cotransduction of the entire circuit. Transduced Jurkat-NFAT cell were diluted with media 1 day after infection and then collected 3 days after infection.

Switch Induction with 4-OHT. Cells were induced with 4-hydroxytamoxifen (4-OHT, Sigma), a metabolite of tamoxifen, in methanol solution. All induction experiments were conducted with 1 μM 4-OHT except for dose response experiments, which were conducted with a 4-OHT concentration range from 10^{-5} to 10 μM. For induction time courses, cells were induced at a starting concentration of 200 000 cells/mL and maintained between 200 000 and 1 200 000 cells/mL with media containing 1 μM 4-OHT. Uninduced cells were also plated and maintained at the same concentrations in inducer-negative media.

Flow Cytometry Sorting and Analysis. Cells were sorted for BFP-positive expression using the SH800 Cell Sorter (Sony. BFP-FL1 channel). To measure circuit switching dynamics and NFAT-GFP expression were measured *via* flow cytometry (Attune NxT Flow Cytometer, Thermo Fisher Scientific. BFP-VL1 channel, PE-YL1 channel, mCherry-YL2 channel, GFP-BL1 channel). Results were analyzed using FlowJo 10.0.7 (FlowJo, LLC). CAR expression in the EXPression Level switch was characterized *via* an mCherry tag directly connected to the CAR. CARs in ON and OFF switches did not contain the mCherry tag, and expression was measured by staining for a myc epitope tag expressed in the extracellular portion of the CAR. Staining was done using a PE-conjugated human c-myc antibody (R&D Systems IC3696P) at a concentration of 10 μL antibody/10^6 cells.

CAR Activation with Plate-Bound Her2 Protein. Target antigen was plated on 96 well, tissue culture-treated flat

bottom plates in Dulbecco's phosphate buffered saline (PBS, Corning) for 2 h at 37 °C. Wells were then washed two times with PBS, and 200 000 cells at a concentration of 1 000 000 cells/mL were plated overnight. To remove supplemental IL-2 prior to plating, primary CD4+ T cells were washed two times and ultimately plated with IL-2-negative media. Cells were plated with or without 4-OHT in accordance with their induction conditions.

IL-2 ELISA. IL-2 production by CD4+ primary T cells was measured using an ELISA kit (BD 550611) according to manufacturer's instruction. 100 μL of supernatant from each sample was collected 19 h postinduction and frozen in −80 °C prior to quantification with the ELISA.

Cell Killing. CD8+ primary T cells were plated with Her2+/GFP+ NALM6 cells at an effector:target ratio of 1:2 on a 96 well, tissue culture-treated flat bottom plates for 20 h. Cells were plated with or without 4-OHT in accordance with their induction conditions. NALM6 cells were counted *via* flow cytometry by gating for live and GFP+ cells. The lack of mycoplasma contamination in NALM6 cells was verified using the MycoAlert Mycoplasma Detection Kit (Lonza LT07−218).

Cell Washing for Memory and Induction Duration. To test memory in switch-expressing CD4+ T cells, cells were induced with 1 μM 4-OHT at a starting concentration of 200 000 cells/mL and maintained between 200 000 and 1 200 000 cells/mL with media containing 1 μM 4-OHT. Uninduced cells were also plated and maintained at the same concentrations in inducer-negative media. A fraction of induced cells was removed from sample 2 days postinduction. To wash away inducer, removed cells were spun down for 5 min at 300g, resuspended in 5 mL inducer-negative, and then spun down again. Cells were resuspended in inducer-negative media and maintained between 200 000 and 1 200 000 cells/mL in inducer-negative media.

To test effect of induction duration, switch-expressing Jurkat T cells were induced with 1 μM 4-OHT at a starting concentration of 800 000 cells/mL. For each day up until 4 days postinduction, a fraction of induced cells were removed. Removed cells were then spun down for 5 min at 300g, washed with 5 mL inducer-negative media, and then resuspended to a concentration of 800 000 cells/mL in inducer-negative media. Induced cells were then diluted 1:2. On day 4, all cells were diluted 1:8, and 8 days postinduction, one last batch of induced cells were washed. Through the rest of the experiment, cells were maintained between 200 000 and 1 600 000 cells/mL.

ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.8b00512.

Supplementary Figures S1−S6 (PDF)

AUTHOR INFORMATION

Corresponding Author
*E-mail: wilwong@bu.edu.

ORCID
Wilson W. Wong: 0000-0001-8394-889X

Author Contributions
D.C. designed and constructed molecular and cellular reagents, conducted experiments, analyzed data, and generated all figures. L.D.C. aided in the construction of molecular and cellular reagents, and B.H.W. aided in initial design and construction of molecular reagents. W.W.W. conceived the project. D.C. and W.W.W. wrote the manuscript.

REFERENCES

(1) Thaxton, J. E., and Li, Z. (2014) To affinity and beyond: Harnessing the T Cell receptor for cancer immunotherapy. *Hum. Vaccines Immunother.* 10, 3313−3321.

(2) Johnson, L. A., Morgan, R. A., Dudley, M. E., Cassard, L., Yang, J. C., Hughes, M. S., Kammula, U. S., Royal, R. E., Sherry, R. M., Wunderlich, J. R., Lee, C. C., Restifo, N. P., Schwarz, S. L., Cogdill, A. P., Bishop, R. J., Kim, H., Brewer, C. C., Rudy, S. F., VanWaes, C., Davis, J. L., Mathur, A., Ripley, R. T., Nathan, D. A., Laurencot, C. M., and Rosenberg, S. A. (2009) Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* 114, 535−546.

(3) Chmielewski, M., Hombach, A., Heuser, C., Adams, G. P., and Abken, H. (2004) T cell activation by antibody-like immunoreceptors: increase in affinity of the single-chain fragment domain above threshold does not increase T cell activation against antigen-positive target cells but decreases selectivity. *J. Immunol.* 173, 7647−7653.

(4) Jena, B., Dotti, G., and Cooper, L. J. (2010) Redirecting T-cell specificity by introducing a tumor-specific chimeric antigen receptor. *Blood* 116, 1035−1044.

(5) Davila, M. L., Riviere, I., Wang, X., Bartido, S., Park, J., Curran, K., Chung, S. S., Stefanski, J., Borquez-Ojeda, O., Olszewska, M., Qu, J., Wasielewska, T., He, Q., Fink, M., Shinglot, H., Youssif, M., Satter, M., Wang, Y., Hosey, J., Quintanilla, H., Halton, E., Bernal, Y., Bouhassira, D. C., Arcila, M. E., Gonen, M., Roboz, G. J., Maslak, P., Douer, D., Frattini, M. G., Giralt, S., Sadelain, M., and Brentjens, R. (2014) Efficacy and toxicity management of 19−28z CAR T cell therapy in B cell acute lymphoblastic leukemia. *Sci. Transl. Med.* 6, No. 224ra25.

(6) Brentjens, R. J., Riviere, I., Park, J. H., Davila, M. L., Wang, X., Stefanski, J., Taylor, C., Yeh, R., Bartido, S., Borquez-Ojeda, O., Olszewska, M., Bernal, Y., Pegram, H., Przybylowski, M., Hollyman, D., Usachenko, Y., Pirraglia, D., Hosey, J., Santos, E., Halton, E., Maslak, P., Scheinberg, D., Jurcic, J., Heaney, M., Heller, G., Frattini, M., and Sadelain, M. (2011) Safety and persistence of adoptively

transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. *Blood 118*, 4817−4828.

(7) Maude, S. L., Frey, N., Shaw, P. A., Aplenc, R., Barrett, D. M., Bunin, N. J., Chew, A., Gonzalez, V. E., Zheng, Z., Lacey, S. F., Mahnke, Y. D., Melenhorst, J. J., Rheingold, S. R., Shen, A., Teachey, D. T., Levine, B. L., June, C. H., Porter, D. L., and Grupp, S. A. (2014) Chimeric antigen receptor T cells for sustained remissions in leukemia. *N. Engl. J. Med. 371*, 1507−1517.

(8) Lee, D. W., Kochenderfer, J. N., Stetler-Stevenson, M., Cui, Y. K., Delbrook, C., Feldman, S. A., Fry, T. J., Orentas, R., Sabatino, M., Shah, N. N., Steinberg, S. M., Stroncek, D., Tschernia, N., Yuan, C., Zhang, H., Zhang, L., Rosenberg, S. A., Wayne, A. S., and Mackall, C. L. (2015) T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. *Lancet 385*, 517−528.

(9) Chakravarti, D., and Wong, W. W. (2015) Synthetic biology in cell-based cancer immunotherapy. *Trends Biotechnol. 33*, 449−461.

(10) Li, H., and Zhao, Y. (2017) Increasing the safety and efficacy of chimeric antigen receptor T cell therapy. *Protein Cell 8*, 573−589.

(11) Kosmrlj, A., Jha, A. K., Huseby, E. S., Kardar, M., and Chakraborty, A. K. (2008) How the thymus designs antigen-specific and self-tolerant T cell receptor sequences. *Proc. Natl. Acad. Sci. U. S. A. 105*, 16671−16676.

(12) Hinrichs, C. S., and Restifo, N. P. (2013) Reassessing target antigens for adoptive T-cell therapy. *Nat. Biotechnol. 31*, 999−1008.

(13) Morgan, R. A., Yang, J. C., Kitano, M., Dudley, M. E., Laurencot, C. M., and Rosenberg, S. A. (2010) Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol. Ther. 18*, 843−851.

(14) Wu, C. Y., Roybal, K. T., Puchner, E. M., Onuffer, J., and Lim, W. A. (2015) Remote control of therapeutic T cells through a small molecule-gated chimeric receptor. *Science 350*, No. aab4077.

(15) Fedorov, V. D., Sadelain, M., and Kloss, C. C. (2014) Novel approaches to enhance the specificity and safety of engineered T cells. *Cancer J. 20*, 160−165.

(16) Kloss, C. C., Condomines, M., Cartellieri, M., Bachmann, M., and Sadelain, M. (2013) Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells. *Nat. Biotechnol. 31*, 71−75.

(17) Sakemura, R., Terakura, S., Watanabe, K., Julamanee, J., Takagi, E., Miyao, K., Koyama, D., Goto, T., Hanajiri, R., Nishida, T., Murata, M., and Kiyoi, H. (2016) A Tet-On Inducible System for Controlling CD19-Chimeric Antigen Receptor Expression upon Drug Administration. *Cancer Immunol. Res. 4*, 658−668.

(18) Hill, Z. B., Martinko, A. J., Nguyen, D. P., and Wells, J. A. (2018) Human antibody-based chemically induced dimerizers for cell therapeutic applications. *Nat. Chem. Biol. 14*, 112−117.

(19) Di Stasi, A., Tey, S. K., Dotti, G., Fujita, Y., Kennedy-Nasser, A., Martinez, C., Straathof, K., Liu, E., Durett, A. G., Grilley, B., Liu, H., Cruz, C. R., Savoldo, B., Gee, A. P., Schindler, J., Krance, R. A., Heslop, H. E., Spencer, D. M., Rooney, C. M., and Brenner, M. K. (2011) Inducible apoptosis as a safety switch for adoptive cell therapy. *N. Engl. J. Med. 365*, 1673−1683.

(20) Straathof, K. C., Pule, M. A., Yotnda, P., Dotti, G., Vanin, E. F., Brenner, M. K., Heslop, H. E., Spencer, D. M., and Rooney, C. M. (2005) An inducible caspase 9 safety switch for T-cell therapy. *Blood 105*, 4247−4254.

(21) Budde, L. E., Berger, C., Lin, Y., Wang, J., Lin, X., Frayo, S. E., Brouns, S. A., Spencer, D. M., Till, B. G., Jensen, M. C., Riddell, S. R., and Press, O. W. (2013) Combining a CD20 chimeric antigen receptor and an inducible caspase 9 suicide switch to improve the efficacy and safety of T cell adoptive immunotherapy for lymphoma. *PLoS One 8*, No. e82742.

(22) Wei, P., Wong, W. W., Park, J. S., Corcoran, E. E., Peisajovich, S. G., Onuffer, J. J., Weiss, A., and Lim, W. A. (2012) Bacterial virulence proteins as tools to rewire kinase pathways in yeast and immune cells. *Nature 488*, 384−388.

(23) Ma, J. S., Kim, J. Y., Kazane, S. A., Choi, S. H., Yun, H. Y., Kim, M. S., Rodgers, D. T., Pugh, H. M., Singer, O., Sun, S. B., Fonslow, B. R., Kochenderfer, J. N., Wright, T. M., Schultz, P. G., Young, T. S., Kim, C. H., and Cao, Y. (2016) Versatile strategy for controlling the specificity and activity of engineered T cells. *Proc. Natl. Acad. Sci. U. S. A. 113*, E450−458.

(24) Rodgers, D. T., Mazagova, M., Hampton, E. N., Cao, Y., Ramadoss, N. S., Hardy, I. R., Schulman, A., Du, J., Wang, F., Singer, O., Ma, J., Nunez, V., Shen, J., Woods, A. K., Wright, T. M., Schultz, P. G., Kim, C. H., and Young, T. S. (2016) Switch-mediated activation and retargeting of CAR-T cells for B-cell malignancies. *Proc. Natl. Acad. Sci. U. S. A. 113*, E459−468.

(25) Kudo, K., Imai, C., Lorenzini, P., Kamiya, T., Kono, K., Davidoff, A. M., Chng, W. J., and Campana, D. (2014) T lymphocytes expressing a CD16 signaling receptor exert antibody-dependent cancer cell killing. *Cancer Res. 74*, 93−103.

(26) Chen, Y. Y., Jensen, M. C., and Smolke, C. D. (2010) Genetic control of mammalian T-cell proliferation with synthetic RNA regulatory systems. *Proc. Natl. Acad. Sci. U. S. A. 107*, 8531−8536.

(27) Torikai, H., Reik, A., Liu, P. Q., Zhou, Y., Zhang, L., Maiti, S., Huls, H., Miller, J. C., Kebriaei, P., Rabinovitch, B., Lee, D. A., Champlin, R. E., Bonini, C., Naldini, L., Rebar, E. J., Gregory, P. D., Holmes, M. C., and Cooper, L. J. (2012) A foundation for universal T-cell based immunotherapy: T cells engineered to express a CD19-specific chimeric-antigen-receptor and eliminate expression of endogenous TCR. *Blood 119*, 5697−5705.

(28) Weinberg, B. H., Pham, N. T. H., Caraballo, L. D., Lozanoski, T., Engel, A., Bhatia, S., and Wong, W. W. (2017) Large-scale design of robust genetic circuits with multiple inputs and outputs for mammalian cells. *Nat. Biotechnol. 35*, 453−462.

(29) Indra, A. K., Warot, X., Brocard, J., Bornert, J. M., Xiao, J. H., Chambon, P., and Metzger, D. (1999) Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. *Nucleic Acids Res. 27*, 4324−4327.

(30) Aelvoet, S.-A., Pascual-Brazo, J., Libbrecht, S., Reumers, V., Gijsbers, R., Van den Haute, C., and Baekelandt, V. (2015) Long-Term Fate Mapping Using Conditional Lentiviral Vectors Reveals a Continuous Contribution of Radial Glia-Like Cells to Adult Hippocampal Neurogenesis in Mice. *PLoS One 10*, No. e0143772.

(31) Loonstra, A., Vooijs, M., Beverloo, H. B., Allak, B. A., van Drunen, E., Kanaar, R., Berns, A., and Jonkers, J. (2001) Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. *Proc. Natl. Acad. Sci. U. S. A. 98*, 9209−9214.

(32) Higashi, A. Y., Ikawa, T., Muramatsu, M., Economides, A. N., Niwa, A., Okuda, T., Murphy, A. J., Rojas, J., Heike, T., Nakahata, T., Kawamoto, H., Kita, T., and Yanagita, M. (2009) Direct hematological toxicity and illegitimate chromosomal recombination caused by the systemic activation of CreERT2. *J. Immunol. 182*, 5633−5640.

(33) Vallier, L., Alexander, M., and Pedersen, R. (2007) Conditional gene expression in human embryonic stem cells. *Stem Cells 25*, 1490−1497.

(34) Takata, Y., Kondo, S., Goda, N., Kanegae, Y., and Saito, I. (2011) Comparison of efficiency between FLPe and Cre for recombinase-mediated cassette exchange in vitro and in adenovirus vector production. *Genes Cells 16*, 765−777.

(35) Long, D. P., Zhao, A. C., Chen, X. J., Zhang, Y., Lu, W. J., Guo, Q., Handler, A. M., and Xiang, Z. H. (2012) FLP recombinase-mediated site-specific recombination in silkworm, Bombyx mori. *PLoS One 7*, No. e40150.

(36) Xue, M., Atallah, B. V., and Scanziani, M. (2014) Equalizing excitation-inhibition ratios across visual cortical neurons. *Nature 511*, 596−600.

(37) Lao, Z., Raju, G. P., Bai, C. B., and Joyner, A. L. (2012) MASTR: A Technique for Mosaic Mutant Analysis with Spatial and Temporal Control of Recombination Using Conditional Floxed Alleles in Mice. *Cell Rep. 2*, 386−396.

(38) Lepper, C., and Fan, C.-M. (2012) Generating tamoxifen-inducible Cre alleles to investigate myogenesis in mice. *Methods Mol. Biol. (N. Y., NY, U. S.)* 798, 297−308.

(39) Lin, J., and Weiss, A. (2003) The tyrosine phosphatase CD148 is excluded from the immunologic synapse and down-regulates prolonged T cell signaling. *J. Cell Biol.* 162, 673−682.

(40) Long, A. H., Haso, W. M., Shern, J. F., Wanhainen, K. M., Murgai, M., Ingaramo, M., Smith, J. P., Walker, A. J., Kohler, M. E., Venkateshwara, V. R., Kaplan, R. N., Patterson, G. H., Fry, T. J., Orentas, R. J., and Mackall, C. L. (2015) 4−1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors. *Nat. Med.* 21, 581−590.

(41) Chang, Z. N. L., Silver, P. A., and Chen, Y. Y. (2015) Identification and selective expansion of functionally superior T cells expressing chimeric antigen receptors. *J. Transl. Med.*, DOI: 10.1186/s12967-015-0519-8.

(42) Riolobos, L., Hirata, R. K., Turtle, C. J., Wang, P. R., Gornalusse, G. G., Zavajlevski, M., Riddell, S. R., and Russell, D. W. (2013) HLA engineering of human pluripotent stem cells. *Mol. Ther.* 21, 1232−1241.

(43) Wang, D., Quan, Y., Yan, Q., Morales, J. E., and Wetsel, R. A. (2015) Targeted Disruption of the beta2-Microglobulin Gene Minimizes the Immunogenicity of Human Embryonic Stem Cells. *Stem Cells Transl. Med.* 4, 1234−1245.

(44) Gornalusse, G. G., Hirata, R. K., Funk, S. E., Riolobos, L., Lopes, V. S., Manske, G., Prunkard, D., Colunga, A. G., Hanafi, L. A., Clegg, D. O., Turtle, C., and Russell, D. W. (2017) HLA-E-expressing pluripotent stem cells escape allogeneic responses and lysis by NK cells. *Nat. Biotechnol.* 35, 765−772.