ENGINEERING GENETIC THERMAL SWITCHES FOR
REMOTE CONTROL OF THERAPEUTIC T CELLS

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ABSTRACT

Adoptive transfer of engineered T cells has shown striking clinical success in treating refractory liquid cancers, yet the inability to spatially regulate T cell activity within select anatomical sites limits efficacy against solid tumors. Inspired by clinical modalities used to heat tumors at depth, we engineer T cells with genetic thermal switches for remote control of transcriptional activity. Synthetic thermal gene switches constructed solely of heat shock elements (HSEs) are tunable and exhibit high specificity to thermal triggers 3-5°C above basal temperature. Using the S7-YB switch, a construct with high specificity for heat induction, we precisely control IL-15 super agonist complex production by donor-derived CAR T cells and preferentially augment T cell proliferation. Together, these factors illustrate the potential of thermal control to augment T cell functions for immunotherapy.

KEYWORDS: synthetic biology, thermal gene switch, remote control, engineered T cells, precision medicine, immunotherapy
INTRODUCTION

Recent advances in engineered T cell therapies have shown promising clinical outcomes in treating cancers that are otherwise unresponsive to conventional therapies. Adoptive transfer of T cells engineered with chimeric antigen receptors (CARs) have led to durable remission of refractory hematological malignancies (Fig. 1). T cells modified to express these synthetic receptors are capable of recognizing antigens expressed on the surface of malignant cells and unleashing potent cytolytic activity.

The clinical success of CAR-T therapy is limited to blood-based cancers however, in large part due to the ability of solid tumors to suppress immune responses. Adjuvant therapies designed to augment T cell effector function can lead to life-threatening complications as a result of cytokine storm. In addition, methods to precisely regulate T cell function, such as through drug-inducible suicide switches or small-molecule activators, lack spatial control and affect engineered cells systemically. Thus, methods to locally augment T cell activity via external cues, such as heat, could increase the efficacy of CAR-T therapy against solid tumors.

By leveraging clinical technologies currently employed to precisely heat patient tumors at depth, we engineer T cells with genetic thermal switches for the remote control of immuno-stimulatory genes (Fig. 2). In the clinic, thermal delivery to tumors has been achieved via modalities such as high intensity focused ultrasound and near-infrared laser heating. Endogenous promoters of heat shock proteins, molecular chaperones that are upregulated in response to mildly elevated temperatures, have been used to control engineered activity of mammalian cells. However, endogenous heat-shock promoters are not heat-specific and contain additional genetic response elements that respond to other cellular stressors such as hypoxia.

Here, we designed a panel of synthetic thermal switches that consist solely of heat-responsive genetic elements. We screened this library for a candidate construct that not only responds
specifically towards thermal cues, but also elicits high levels of gene expression upon heat treatment. We demonstrated that synthetic thermal switches are unresponsive towards model cellular stressors such as hypoxia and toxic heavy metals. Finally, we engineered donor-derived CAR-T cells with thermal circuits incorporating the immunostimulatory IL-15 super agonist complex, and precisely augment T cell proliferation using thermal cues.

Figure 1. Schematic representation of the adoptive transfer of engineered T cells for cancer therapy.
METHODS

Switch design and plasmid construction. The Hspa6 - Gluc – P2A – emerald GFP SFFV-mCherry plasmid was constructed as described previously\(^\text{19}\). The constitutive EF1-\(\alpha\) anti-CD19 CAR plasmid encoding the CD19-specific CAR was kindly provided by Dr. Krishnendu Roy (Georgia Institute of Technology). Core promoters were isolated from genomic DNA and truncated immediately upstream of their previously described TATA boxes\(^\text{24-26}\). The sequence for YB_TATA was obtained from literature\(^\text{27,28}\). To create switch constructs, 2-7 heat shock elements (5’ – nGAAAnnTTCnnGAAAn – 3’) were arrayed upstream of selected core promoters and flanked by restriction sites. The Gluc – P2A – IL-15 SA sequence was derived from literature\(^\text{29}\). All DNA fragments were chemically synthesized (Integrated DNA Technologies). Constructs were cloned into the HSPA6-Gluc-P2A emerald GFP plasmid and inserted in place of the HSPA6 promoter via SphI and XhoI sites by restriction enzyme digestion and ligation. To construct the S7-YB IL-15SA \(\alpha\)CD19 CAR plasmid, Gluc-P2A-IL-15 SA was inserted into the EF1-\(\alpha\) anti-CD19 CAR plasmid via AgeI and NotI sites by restriction enzyme digestion and ligation. Plasmid DNA was purified using a Plasmid Maxi Kit (Omega Bio-Tek #D6926-01) and sequenced-verified (Eurofins Genomics, Louisville, KY).

Culture of primary human T cells and cell lines. Jurkat cells (ATCC) were cultured in RPMI-1640 Medium (Life Technologies #11875119) supplemented with 10% heat-inactivated fetal bovine...
serum (Fisher #16140071) and 10 U/ml Penicillin-Streptomycin (Life Technologies #15140-122). Primary human CD3+ T cells were obtained from anonymous donor blood following apheresis (AllCells) and were cryopreserved until subsequent use. Two days prior to lentiviral transduction, primary human CD3+ T cells were thawed and cultured in human T cell medium, consisting of X-VIVO 10 (Lonza #04-380Q), 5% human AB serum (Valley Biomedical #HP1022), 10 mM N-acetyl L-Cysteine (Sigma #A9165), and 55 µM 2-mercaptoethanol (Sigma #M3148-100ML) supplemented with 50 units/ mL human IL-2 (Sigma #11147528001).

**Lentiviral production and primary T cell transduction.** Lentivirus was produced via transfection of HEK 293T cells (ATCC) using psPAX2 (Addgene #12260) and pMD2.G (Addgene #12259). Viral supernatant was concentrated using PEG-it virus precipitation solution (System Biosciences LV825A-1). For viral transduction, primary human T cells were thawed for 24 hours and activated with Dynabeads Human T-Activator CD3/CD28 at a 3:1 bead:cell ratio (Life Technologies #111.31D). To transduce the activated T cells, concentrated lentivirus was added to non-TC treated 6-well plates which were coated with retronectin (Takara #T100B) according to manufacturer’s instructions and spun at 1200 x g for 90 min at room temperature. Following centrifugation, viral solution was aspirated and 2 mL of human T cells (250,000 cells / mL) in human T cell media containing 100 units / mL hIL-2 were added and spun at 1200 x g for 60 min at 37 °C and moved to an incubator. Cells were incubated on virus-coated plate for 24 hours prior to expansion. Dynabeads were removed 7 days after T cell activation.

**Flow cytometry.** CAR+ cells were labeled with biotinylated human CD19 (VWR #10312-528). Streptavidin-PE (ThermoFisher #S866) was subsequently added for CAR expression quantification. Samples were resuspended in DPBS (ThermoFisher # 14200166) supplemented with 25 mM HEPES (Fisher # 15630080), 1% BSA (Sigma #A7030), and 5 mM EDTA (Fisher Scientific #AM9260G). Samples were analyzed using a BD LSR Fortessa Cell Analyzer or a BD C6 Accuri. Fluorescence-activated cell sorting (FACS) using a BD FacsAria Fusion Cell Sorter.
was performed to isolate CAR\textsuperscript{high} transduced cells (IL-15SA assays) and mCherry\textsuperscript{high}emGFP\textsuperscript{low} transduced cells (luciferase assays). Flow data was analyzed via the FlowJo Data Analysis Software.

\textit{Luciferase assays.} Jurkats and primary human CD3+ T cells transduced with thermal luciferase circuits were heated in a thermal cycler (Biorad). Immediately after heating, cells were transferred to a 96-well plate and incubated at 37°C. For hypoxia and heavy metal studies, cobalt chloride and cadmium chloride treated cellular supernatant was sampled for luciferase activity as specified. Luminescence was measured using a Cytation 5 plate reader (BioTek) and a Pierce Gaussia Luciferase Glow Assay Kit (ThermoFisher #16161) according to manufacturer’s instructions.

\textit{Quantification of IL-15 SA production.} Transduced primary T cells were heated in a thermal cycler (Biorad) and transferred to a 96-well plate for 24 hr. IL-15 SA production in cellular supernatant was measured using a Cytation 5 plate reader (BioTek) and the HIL-15/IL-15 RA Duoset Kit (Fisher #DY6924) according to manufacturer’s instructions.

\textit{T cell proliferation assays.} Primary human CD3+ T cells transduced with the S7-YB IL-15SA EF1a anti-CD19 CAR plasmid were heated in a thermal cycler and transferred to a 96-well plate. Wild-type primary human CD3+ T cells were labelled with carboxyfluorescein succinimidyld ester (CFSE) and added to heated S7-YB cells at a 1:1 ratio. Proliferation was quantified via flow cytometry.

\textit{Statistical Analysis.} All results are presented as mean, and error bars show SEM. Statistical analysis was performed using statistical software (GraphPad Prism 6; GraphPad Software). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=not significant.
RESULTS

Engineering a genetic thermal switch

The heat shock response is a conserved stress response that leads the synthesis of heat shock proteins (HSPs), molecular chaperones that aid in the repair of damaged proteins\(^{30-32}\). This pathway is primarily regulated by Heat Shock Factor 1 (HSF1), a transcription factor that exists as an inactive monomer under basal temperatures. In response to thermal stress, HSF1 associates into a high affinity homotrimer and binds to heat shock response elements (HSEs) within endogenous heat-shock promoters to initiate the transcription of target genes. However, the heat-shock response is also induced by other cellular and environmental stressors. Response elements such as those for hypoxia and heavy metals have been identified within the sequences of heat-shock promoters\(^{22-24}\). In addition, the response characteristics of endogenous promoters is regulated by the core promoter, a minimal sequence that consists of the TATA box and 5’ UTR\(^{33,34}\). The core promoter directs the assembly of the pre-initiation complex (PIC), a complex of transcription factors and RNA polymerase II\(^{34}\). Due to the capacity of endogenous heat-shock promoters to integrate multiple stress-dependent signals, we sought to construct synthetic thermal gene switches consisting solely of HSEs and a core promoter. We aimed to identify switches with not only high specificity and responsiveness to thermal cues, but also minimal activity at basal temperatures.

To do so, we first identified candidate core promoters that had high inducibility following heat treatment. Primary T cells were incubated at elevated temperatures (40-42°C, 15 min) and the transcriptional activity of heat-shock promoters was profiled via quantitative PCR (Fig. 3). In comparison to basal expression at 37°C, Hspb1 (Hsp27), Hspa1a, and Hspa1b exhibited high induction of mRNA expression following heat-treatment at 42°C (1790-fold, 497-fold, and 221-fold, respectively). Due to its high heat inducibility, the Hsp27 core promoter was then isolated from genomic DNA and truncated at the TATA box as previously identified\(^{26}\).
To create synthetic thermal gene switches, 2 to 7 repeating units of HSEs (5' – nGAAnnTAAn – 3') were serially arrayed upstream of the Hsp27 core promoter to generate a repertoire of S2-27 to S7-27 switches (Synthetic n-repeating heat shock elements – Hsp27 core promoter) (Fig. 4a). Switch constructs were cloned upstream of a Gaussia luciferase (Gluc) reporter and Jurkat T cells were transduced to evaluate thermal switch activity. In response to heat treatment at 42°C for 60 minutes, we observed that as few as two HSEs sufficiently induced luciferase expression, and further multimerization of HSEs increased switch (Fig. 4b). In the S7-27 switch, a construct consisting of 7 HSE repeats, luciferase expression increased up to ~400 fold in comparison to cells treated at 37°C (Fig. 4c).

Figure 3. Heat-shock promoter screen. Transcriptional activity of heat-shock promoters as quantified by quantitative PCR, n=3.
In human primary CD3+ T cells transduced with the S7-27 switch, heat treatment at either increasing temperatures (40-42°C) or longer durations (15-30 min) resulted in an increase in luciferase activity in a dose dependent manner (Fig. 4d). To evaluate the activation kinetics of the S7-27 switch, transduced human CD3+ cells were subjected to heat-shock, and luminescence was sampled at respective time points. Following heat-shock, we observed an initial increase in Gluc expression after 3 hours and maximum activity after 6 hours, in which luminescent signal was elevated by 12-fold in comparison to unheated controls (Fig. 4e). To evaluate the duration of activity of the S7-27 switch following heat-treatment, we repeatedly sampled and replaced cellular supernatant after maximum switch activity was attained (Fig. 4f). At respective time-points, we observed no appreciable increase in luciferase activity 6 hours post heat-shock.

**Figure 4. Design and characterization of Hsp27-derived synthetic thermal switches.** (a) Schematic representation of Hsp27-derived synthetic thermal switches. (b) Luciferase activity in Jurkat T cells transduced with switch constructs of multimerized HSEs, n=3, errors bars=SEM. (c) Fold induction of normalized luminescence, n=3, error bars=SEM. (d) Luciferase activity of S7-27 transduced CD3+ T cells following respective heat treatments, n=3, error bars=SEM, ****p<0.0001, Two-way ANOVA, Sidak’s multiple comparisons test. (e) Kinetic traces of S7-27 switch activity following 30 min heat-shock, n=3, error bars=SEM, ****p<0.0001, Two-way ANOVA, Tukey’s multiple comparisons test. (f) Luminescent trace of S7-27 activity after maximal signal is attained following 30 min heat-shock at 42°C, n=3, error bars=SEM, ****p<0.0001, Two-way ANOVA, Tukey’s multiple comparisons test.
**Synthetic thermal switches are specific to heat-shock**

In prior studies, endogenous heat-shock promoters such as the human Hspa6 promoter have been used to enable heat-mediated transgene expression in mammalian systems\textsuperscript{19-21}. Upon incubation at basal temperature (37°C) for up to 60 minutes, Jurkat T cells transduced with the endogenous Hspa6 promoter exhibited increased expression of luciferase activity (Fig. 5a). In response to cold-shock (4-35°C), the Hspa6 promoter elicited significantly higher luciferase activity in comparison to synthetic switch constructs across treatments (Fig. 5b). Prior sequence analysis of the Hspa6 promoter has identified genetic response elements for cyclic AMP, hypoxia, and cadmium\textsuperscript{24}. We hypothesized that these response elements were responsible for the nonspecific activation of the Hspa6, and that S3-27 – S7-27 synthetic switches would not respond to orthogonal stressors, regardless of HSE number.

We then sought to evaluate the activity of synthetic switches in response to model cellular stressors, such as hypoxia and environmental toxins\textsuperscript{35-37}. To assess switch activity in response to hypoxic conditions, we treated transduced Jurkats at different concentrations (0-1 mM) of cobalt chloride, a chemical inducer of hypoxia inducible factor 1 α (HIF 1 α)\textsuperscript{38}. We observed significantly elevated Hspa6 promoter activity across all treatments, whereas luminescent signal of synthetic constructs remained comparable to that of wild-type controls (Fig. 5c).

To evaluate switch activity in response to heavy metals, we treated transduced cells at different concentrations of cadmium, an environmental carcinogen found in fertilizers and plastics\textsuperscript{39,40}. Following treatment of cadmium chloride\textsuperscript{41} (0-500 μM) we similarly observed no significant increase in luciferase expression from synthetic thermal switches across treatments, whereas the Hspa6 promoter is activated at cadmium concentrations greater than 125 μM (Fig. 5d).
Figure 5. Activity of synthetic thermal switches in response to model cellular stressors. (a) Kinetic trace of transduced Jurkat T cells following incubation at 37°C, n=3, error bars=SEM, ****p<0.0001, Two-way ANOVA, Tukey’s multiple comparisons test. (b) Luminescent trace of transduced Jurkat T cells following hypothermic stress, n=3, error bars=SEM, *p<0.05, **p<0.01, ****p<0.0001, Two-way ANOVA, Tukey’s multiple comparisons test. (c) Luminescent trace of transduced Jurkat T cells following respective treatment of CoCl$_2$, n=3, error bars=SEM, ****p<0.0001, Two-way ANOVA, Tukey’s multiple comparisons test. (d) Luminescent trace of transduced Jurkat T cells following respective treatment of CdCl$_2$, n=3, error bars=SEM, *p<0.05, ****p<0.0001, Two-way ANOVA, Tukey’s multiple comparisons test.
Core promoter dictates thermal switch activity

While S2-27 to S7-27 synthetic switches exhibited similar activation kinetics (Fig. 6a) and higher specificity towards thermal triggers, luciferase activity of synthetic switches was 2-fold lower in comparison to that of the Hspa6 promoter in transduced primary human CD3+ T cells (Fig. 6b).

![Graph](image)

**Figure 6. Comparison of HSPA6 and S7-27 switch activity in human primary T cells** (a) Kinetic trace of transduced primary human CD3+ following 30 min heat-shock at 42°C. n=3, error bars=SEM. (b) Luminescent traces of transduced human primary CD3+ T cells following 30 min heat-shock at 42°C, n=3, error bars=SEM, **p<0.01, Student’s t-test.

To develop a switch construct with a higher magnitude of activity, we varied the core promoter of the S7-27 switch with those of other highly inducible promoters (Fig. 7a). Hspa1a, Hspa1b, Hspa6, and the synthetic YB_TATA core promoter described previously were truncated downstream a sequence of 7 HSEs to generate S7-A1B, S7-A6, and S7-YB, respectively. Heat treatment (42°C, 30 min) of transduced primary T cells resulted in variable Gluc expression that was dependent on core promoter sequence. Notably, S7-27 and S7-YB switches exhibited negligible activity comparable to that of wild-type controls when treated at 37°C (Fig. 7b). Following heat-treatment, cells transduced with the S7-YB switch elicited a ~60-fold increase in switch activity and luciferase expression comparable to that of the Hspa6 (Fig. 7b). As S7-27 and S7-A1B
switches were derived of murine promoters identified in the qPCR screen (Fig. 3), we hypothesized that choice of core promoter sequence may correspond to heat-responsiveness across species. Following heat-shock (42°C, 15 min) of transduced primary murine CD8+ T Cells, we observed a ~3-fold and ~5-fold increase in emGFP expression in the S7-27 and S7-YB switches, respectively (Fig. 7c). In comparison, emGFP expression from the Hspa6 promoter increased by less than 2-fold.

Due to the negligible basal activity of the S7-27 and S7-YB switches, we were interested to see if febrile conditions would induce thermal switch activity. Transduced primary human CD3+ T cells were treated in conditions characteristic of fevers (39-40°C, 24 hours). Cellular supernatant was sampled 48 hours following initial heat treatment, and negligible switch activity following incubation at temperatures below 40°C was observed (Fig. 7d).

Thermal IL-15 super agonist circuits augment T cell proliferation

Due to the degree of cellular control afforded through the S7-YB switch, we hypothesized that we could use thermal cues to precisely induce the expression of therapeutic genes by T cells. We engineered donor-derived anti-CD19 CAR-T cells to conditionally express the IL-15 super agonist (IL-15 SA) (Fig. 8a), a fusion protein that has demonstrated therapeutic efficacy in preclinical studies42,43, but is also associated with systemic immunotoxicity44,45. Incubation of S7-YB CAR-T cells at 37°C exhibited undetectable levels of IL-15 SA by ELISA (Fig. 8b). By contrast, IL-15 SA was highly expressed by S7-YB CAR-T cells following heat treatment (42°C, 30 min) (Fig. 8b). As expected, IL-15 SA expression of S7-YB CAR-T cells increased following heat-shock at 42°C for increasing durations (Fig. 8c). To determine the effect of heat-induced IL-15 SA on T cell proliferation, we labeled wild-type primary CD3+ T cells with CFSE, a fluorescent molecular dye, and quantified T cell proliferation based on the decrease of fluorescent signal. After 6 days, wild-type cells cocultured with heated S7-YB CAR T cells (42°C, 30 min) exhibited enhanced proliferation in comparison to unheated controls (Fig. 8d,e).
Figure 7. Modulation of synthetic thermal switches via core promoter sequence. (a) Schematic representation of synthetic thermal switches incorporating different core promoters. (b) Luciferase activity of primary human CD3+ T cells transduced with S7-core promoter switches following heat-shock, n=3, error bars=SEM., Two-way ANOVA, Tukey’s multiple comparisons test (c) Flow cytometry plots of primary murine CD8+ T cells transduced with S7-core promoter switches following heat-shock (d) Luciferase activity of switch constructs in response to febrile conditions, n=3, error bars=SEM, Two-way ANOVA, Tukey’s multiple comparisons test.
Figure 8. Thermal control of IL-15 SA mediated T cell expansion via the S7-YB switch. (a) Schematic representation of thermally regulated IL-15 SA production by S7-YB CAR-T cells. (b) Protein expression of heat-triggered IL-15 SA in S7-YB CAR-T cells as quantified by ELISA 24 hr post heat-treatment, n=3, error bars=SEM. (c) Protein expression of heat-triggered IL-15 SA in S7-YB CAR-T cells following thermal cues of increasing duration, n=3, error bars=SEM. (d) Representative histograms of cell proliferation dyed (CFSE) wild-type human CD3+ T cells following coculture with heated S7-YB CAR-T cells. (e) Proliferation of wild-type human CD3+ T cells after 6 days of coculture with heated S7-YB CAR-T cells, n=3, error bars=SEM, ***p<0.001, Student’s t-test.
DISCUSSION & CONCLUSION

Engineered T cell therapies have shown limited efficacy for the treatment of solid tumors and the subsequent administration of immune adjuvants can lead to systemic toxicities. In this study, we designed a class of genetic thermal switches for the remote control of engineered T cell therapies. Synthetic thermal gene switches are modular, have high specificity to thermal triggers, and enable precise control of cellular activity.

In previous work, we constructed thermal luciferase circuits driven by the Hspa6 promoter and remotely controlled the activity of engineered T cells in a murine model\textsuperscript{19}. Here we demonstrated that the Hspa6 promoter exhibits increased basal activity and is activated by non-thermal cellular stressors, such as hypoxia or heavy metals. These cellular stressors are present in organs such as the heart, liver, and kidney\textsuperscript{49-52}; thus, endogenous promoters may aberrantly activate engineered cells \textit{in vivo} and induce off-target toxicity. In contrast, synthetic thermal switches consisting solely of HSEs displayed negligible activity following exposure to these conditions. Furthermore, synthetic thermal switches are activated within a narrow temperature window (40-42°C) and are silent at temperatures characteristic of both hypothermia and fevers. We anticipate that the specificity of synthetic thermal switches towards relevant heat shock may offer an enhanced safety profile \textit{in vivo}.

Through the variation of HSEs and core promoter sequences, we demonstrated the tunability of switch activity in response to thermal cues. While increasing multimerization of HSEs led to increasing amounts of reporter expression, the choice of core promoter led to large variations in switch characteristics. Surprisingly, core promoter sequences of highly inducible heat-shock promoters did not correlate to higher amounts of gene expression in synthetic thermal switches. Absence of structural interactions that promote PIC assembly\textsuperscript{46-48}, such as DNA looping, between upstream regulatory regions may in part explain this discrepancy. In contrast, the YB_TATA, an artificial core promoter used to construct the S7-YB switch, yielded strong inducibility and low
basal activity in both primary human and murine T cells. Prior studies suggest that the low basal activity of the YB_TATA is due to reduced levels of GC content immediately (-7 bp) upstream of the TATA box, which minimizes basal binding of the PIC without impairing activated transcription$^{56,57}$.

Using the S7-YB thermal switch, we show precise regulation of therapeutic T cell function, including cytokine production and proliferation of bystander T cells. In contrast to small-molecule based systems, in which engineered cells are systemically affected following the administration of the inducer$^{13-16}$, the thermal switch platform enables precise cellular control – at depth – using clinical modalities such as focused ultrasound or near-infrared laser heating$^{16-18}$. The specificity of the S7-YB switch towards a narrow range of thermal cues allowed for the delivery of potent immunostimulatory biologics, such as IL-15 SA, that have demonstrated toxicity in vivo$^{42,43}$. Moving forward, the S7-YB switch may be used to safely deliver other therapeutic payloads, such as high-affinity CARs$^{56,57}$ or bispecific T cell engagers (BiTEs)$^{58,59}$, to improve clinical outcomes against solid tumors. In a broader context, synthetic thermal switches provide an orthogonal mechanism of cellular regulation and can be used alongside both exogenous and autonomous systems$^{60-62}$ for exquisite control of cellular therapies.
REFERENCES


