UNIVERSITY OF CALIFORNIA SAN DIEGO

Developing thermo-activatable genetic circuits for immunotherapy

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by

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The Thesis of Chunyang Song is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

Developing thermo-activatable genetic circuits for immunotherapy

by

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In recent years, CAR-T cell therapy has revolutionized cancer immunotherapy, but there are still major obstacles preventing its broader applications in treating solid tumors. Challenges including on-target off-tumor toxicities increased the risks associated with CAR-T cell therapy and currently proposed strategies still lacked precise spatiotemporal control for CAR-T cell

activation. To address this issue, we identified a calcium ion channel TRPV1 that is activated by heat and integrated it into an AND-gate inducible genetic circuits in HEK293T cells, to convert the TRPV1 thermal-activation into transcriptional activities. We first demonstrated heat-inducible luciferase reporter gene expression in engineered HEK293T cell line. We further engineered HEK293T cells to present tumor specific antigen CD19 upon heat activation and study the killing by anti-CD19 CAR-T cells. We showed that engineered HEK293T cells presented CD19 upon thermal-activation and were recognized and attacked by anti-CD19 CAR-T cells, demonstrating the feasibility of the system in immunotherapy. In the future, with a well-established focused ultrasound system, we hope to induce temperature changes in the confined volume of tissue *in vivo* to address the on-target off-tumor toxicities associated with current CAR-T cell therapies.

1. Introduction

1.1 Transient receptor potential cation channel subfamily V, member 1 (TRPV1)

Transient receptor potential cation channel subfamily V member 1 (TRPV1), previously known as vanilloid receptor 1 (VR1), is a nonselective ligand-gated ion channel with moderate Ca^{2+} permeability [1]. Expression of the receptor is enriched in immune cells, sensory neurons, and some tumor cells [2]. TRPV1 channels possess crucial roles in the perception of pain, detection of noxious environmental stimuli, and regulation of body temperature in human [3].

TRPV1 is tetrameric, with each homo-subunit consisting of 6 transmembrane domains (S1-S6) [4]; the additional hydrophobic loop between S5 and S6 forms a pore domain and is important for TRPV1 channel opening and cation influx [5]. Both the N- and C-terminus of each monomer locate in the cytosolic region; the N-terminus of TRPV1 contains multiple phosphorylation sites for binding ATP while the C-terminus contains the TRP domain and is involved in heat sensitivity [6]. Heat-dependent TRPV1 opening comprises 2 steps: universal conformational changes preparing the channel for opening, followed by outer pore conformational changes leading to channel opening [7]. It is experimentally verified that the TRPV1 channel is activated by noxious heat greater than 43 °C and allows up to 10 times of calcium influx into the cytoplasm [8].

TRPV1, also called the capsaicin receptor, is activated by capsaicin (the most notable type of vanilloid ligand) [9]. Capsaicin, derived from plants of the *Capsicum* genus, is the active chemical substance that gives people a burning sensation from chili peppers [10]. Capsaicin forms hydrogen bond with cytosolic region of S3 and S4 of TRPV1, slightly changes the configuration of tetramer, and lowers the temperature threshold for opening the channel [4][11].

Overall, being a heat and ligand-gated ion channel, TRPV1 responds quickly and repeatedly to stimuli, making itself a promising component for thermo-activatable applications.

1.2 Nuclear factor of activated T-cells (NFAT)

Calcium signaling is crucial to many intracellular and extracellular processes, such as neurotransmitter release in synapses, chondrogenic differentiation, and skeletal muscle contraction [12]. In particular, calcium signaling is of utmost importance to immune cells. One of the major calcium signaling in T cells is the calcium-calmodulin-calcineurin pathway, which is primarily governed by intracellular calcium concentration [13]. If cytosolic calcium concentration increases as a result of calcium uptake through ion channels on the plasma membrane or release from the endoplasmic reticulum store, secondary messenger calcium ion will bind to the ubiquitous calcium-modulated protein - calmodulin and activates it. Calcium-saturated calmodulin will then bind to the regulatory element of intermediate calcium messenger calcineurin and activates its phosphatase activity [14].

Calcineurin is a serine/threonine protein phosphatase which will be activated upon calcium influx and calmodulin activity to proceed with downstream signaling and regulate T cell activation in mammals [15]. Nuclear factor of activated T cells (NFAT), a group of transcriptional factors that activate the expression of genes important for T-cell activation, differentiation, and function, is regulated by the phosphatase activity of calcineurin [16]. Activated calcineurin dephosphorylates NFAT; dephosphorylated NFAT becomes activated and translocate into the nucleus, binds to NFAT response element (RE), and induces NFAT-mediated transcription [17].

4 out of 5 NFAT isoforms (NFAT1-4) contain a calcineurin binding domain, which allows them to be sensitive to calcium concentration [18]. Previously, people believed that only immune cells are under NFAT regulation, but recent studies show that ubiquitous NFAT expression was indeed observed in most human tissues [18]. Notably, NFAT dephosphorylation by calcineurin is

fast and reversible, making it a promising component in designing inducible synthetic genetic circuits [19].

1.3 Chimeric antigen receptor T-cell therapy

Chimeric antigen receptors (CAR) are engineered fusion protein molecules consisting of a single-chain variable fragment (scFv) of an antibody against the tumor specific antigen (TSA), an extracellular spacer fused to the transmembrane domain of T cell receptor (TCR), and a cytoplasmic costimulatory domain [20][21]. T cells engineered to express CARs on their cell membranes can recognize target antigens on tumor cells via the scFv domain, resulting in T cell activation and attack on tumor cells [22]. In CAR-T cell therapies, T cells are taken from patients themselves or healthy donors, engineered with CARs, and given back to the patients [23]. This form of personalized treatment achieved significant success in treating B cell malignancies, especially B cell acute lymphoblastic leukemia (B-ALL), with only 10% of relapse [22].

Although CAR-T cell therapy has revolutionized cancer treatment, there are obstacles preventing broader applications in treating cancers. One common problem for both leukemia and solid tumors is antigen escape, where tumor cells downregulate their surface target antigen [24]. Particularly, cells from solid tumors express a lower level of the target antigen, making recognition by CAR extremely difficult [25]. In addition, on-target off-tumor toxicities appear to be the greatest risk of current CAR-T cell therapies. Tumor target antigens, such as CD19, are also expressed on many normal cell membranes [26]. As a result, CAR-T cells targeting the antigen will kill tumor cells and normal cells in a non-discriminating manner, leading to the significant defect which is commonly referred to as on-target off-tumor toxicity. Particularly in some solid

tumors, target antigen expression level in tumor cells is only less than a magnitude higher compared to normal cells [27].

Focusing on increasing the specificity of CAR-T cell attack, especially for solid tumor applications, researchers came up with many solutions. Suicide switch, split-CAR, Synnotch, and other synthetic receptors were developed, but their drawbacks include downregulation or loss of CAR activity, poor spatial specificity, and diffusion-limited temporal resolution [28][29][30][31]. Optogenetics, using light to control molecular interactions, has shown much promise in controlling T cell activation. Light-controlled protein-protein interactions effectively activated CAR-T cells with high spatiotemporal control [32]. However, light has limited penetration depth for targeting deep tumor tissue, and there still lacks an effective way to control CAR-T cell activation noninvasively with high spatiotemporal resolution.

1.4 Tetracycline-inducible AND-gate genetic circuits

Following the above discussion, our proposed method for combating on-target off-tumor toxicity of CAR-T therapy is also controllable inducible genetic circuits but uses components that allow precise spatiotemporal control of transcription initiation. We incorporated TRPV1-mediated calcium influx and NFAT-mediated transcriptional activation into our inducible circuit because as mentioned in chapters 1.1 and 1.2, TRPV1's response to thermal activation is fast, and NFAT dephosphorylation by calcineurin is fast and reversible, making them promising components in designing a synthetic genetic circuit.

Localized heat-induced calcium influx through TRPV1 and consequent NFAT translocation is one component that spatially regulates transcription in our system design. In order to achieve more precise control of the gene of interest (GOI) expression with low basal leakage,

another component we incorporated in the system design is a tetracycline-controlled transcriptional activation system. In the original tetracycline-off system (Tet-Off), a tetracycline-controlled activator (tTA) was created by combining the tetracycline repressor (TetR) with a transcriptional activator VP16 [33]. Without tetracycline or its analogs such as doxycycline, TetR will bind to the tetracycline response element (TRE) and VP16 activates transcription. In contrast, in the presence of tetracycline, tetracycline will preferentially bind to TetR, therefore stopping the tTA unit from binding to the TRE region and preventing GOI transcription. Afterward, a new tetracycline-on system (Tet-On) was developed, where a silenced genetic circuit could be turned on in the presence of tetracycline. The new reverse tetracycline-controlled trans-activator (rtTA) was created by fusing reverse tetracycline repressor (rTetR), a mutated form of TetR, with VP16. Thus, only in the presence of tetracycline, rTetR binds to the TRE region, and VP16 again activates transcription [34][35].

Although Tet-On and Tet-Off systems have been widely used in the field of molecular engineering, certain levels of leaky expression of GOI have always been reported [36][37]. Therefore, in our system, to minimize basal gene expression and increase the contrast between control and treatment groups, an AND-gate system requiring simultaneous 2-factor activation was designed. More specifically, both calcium influx and tetracycline are needed for GOI expression in our system design. With localized TRPV1 thermal activation and tetracycline treatment of the time window we are interested in, we can achieve tight and precise spatiotemporal control of GOI expression.

In general, we came up with 2 ideas, addressing CAR-T cells and tumor cells respectively. Our aims are to 1) either control CAR expression in T cells such that the CARs are only expressed when they are in the correct tumor environment, or 2) specifically engineer tumor cells with target

antigens such that only the engineered cells are recognized by constitutive CAR-T cells and killed. Both strategies require tightly controlled inducible GOI expression with high spatiotemporal resolution.

2. Materials and Methods

2.1 Constructs and plasmids

All plasmid templates were obtained from Dr. Chi Woo Yoon. Standard molecular cloning techniques including polymerase chain reaction (PCR), restriction enzyme digestion, Gibson assembly, ligation, etc. were applied to construct plasmids containing rtTA-m1NFAT, TRE-Fluc-PGK-Rluc, TRE-tCD19-EGFP, and TRPV1. from existing templates in the Wang Lab. Primers for PCR were ordered from Integrated DNA Technologies.

2.2 Cell culture and reagents

HEK293T, Lenti-X 293T, Jurkat T and peripheral blood mononuclear cells (PBMCs), were offered by Dr. Chi Woo Yoon. HEK293T and Lenti-X 293T cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin and streptavidin. Jurkat T cells were cultured in RPMI-1640 (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin and streptavidin. PBMCs were cultured in X-VIVO 15 medium (Lonza) supplemented with 5 % FBS (Gibco) and 1% penicillin and streptavidin and 0.1% Interleukin 2 (Peprotech). All cells were maintained in incubator at 37 °C with 5% CO_2.

Doxycycline, ionomycin, and capsaicin were purchased from ThermoFisher, and blasticidin was purchased from InvivoGen.

2.3 Transient transfection

1 day prior to transfection, 0.4/0.8 x 10^6 HEK293T cells were seeded in a 12/6-well plate. Transfection of 500/1000 ug TRPV1 plasmids was performed using Lipofectamine™ 3000 Transfection Reagent (ThermoFisher) under the manufacturer's instruction. On the following day,

transfected cells were passed to fibronectin-coated 96-well plates for Firefly luciferase and Renilla luciferase assay or 12-well plates for EGFP flow cytometry measurements.

10 x 10^6 Jurkat cells were electroporated with 10 ng of TRPV1 plasmid using 4D-Nucleofactor (Lonza) according to the manufacturer's instructions.

2.4 Cell lines and lentiviral infection

Pseudo-typed lentivirus was produced from Lenti-X 293T cells co-transfected with transgene plasmid, viral envelope vector (VSV-G), and packaging vector (deltaR8.91) using Profection™ Mammalian Transfection System (Promega) according to manufacturer's instructions. Viral supernatant was collected 48 hours after the transfection, filtered, and concentrated using PEG-it Virus Precipitation (System Biosciences). 18 hours later, the concentrated virus was centrifuged for 30 minutes at 4 °C and resuspended in PBS.

One day before infection, HEK293T cells were seeded in a 12-well plate (0.1 x10^6 and 0.05 x10^6 cells respectively). 5-20 ul of the concentrated virus were added to each well. 2 days after infection, infected cells expressing fluorescent protein markers were sorted using SH800S Cell Sorter (Sony). Infected cells expressing blasticidin were selected using media supplemented with 5-10 ug/ml of BSD (InvivoGen).

2.5 Thermal activation and drug treatment

All cell lines were thermally activated to evaluate TRPV1 channel thermo-activation potential. 1 day after transfection, HEK293T cell lines transiently transfected with TRPV1 were passed to fibronectin-coated 96-well plates at an appropriate density (70%). Jurkat T cell line transfected with TRPV1 by electroporation and T cell line were passed to 96-well plates on the

experimental day. Cells were treated with conditions including negative control, doxycycline (100nM), ionomycin (10 ug/ml), doxycycline and ionomycin, doxycycline, and capsaicin (50 uM), heat, heat with doxycycline, heat with doxycycline and capsaicin. Heating for all cells (HEK293T cells) was performed in water bath at 43.5 °C for 2 minutes, transferred to 37 °C incubator to cool down for 3 minutes for a total of 6 times. Heating pattern experiment was performed using C1000 Touch Thermal Cycler (Biorad) at 43°C for 30s, cool down at 37°C for 4.5 minutes; the cycle was repeated 2/4/6/8 times.

1 hour after drug and/or heat treatment, media containing drugs was aspirated, and cells were replenished with fresh media. Respective assays were carried out 6 hours after thermal activation and drug treatment.

2.6 T cell co-culture killing assay

Anti-CD19 CAR-T cells were activated using Dynabeads (ThermoFisher) 7 days prior to killing assay. HEK293T cell line expressing rtTA-m1NFAT and TRE-tCD19-EGFP were transiently transfected with TRPV1, treated with drug mentioned, and thermally activated. 1 hour after treatment, activated anti-CD19-CAR T cells were added to HEK293T cells at a 1:1 effector to target ratio. After 24 hours, HEK293T cell viability was measured by ATP luciferase assay.

2.7 Luciferase reporter assay

6 hours after thermal activation and drug treatment in HEK293T and Jurkat cell lines expressing TRE-Fluc-PGK-Rluc, Renilla and Firefly luciferase assay were performed using Dual-Glo Luciferase Assay System (Promega) according to manufacturer's instructions. Luminescence quantification was measured using Tecan Infinite M1000 Pro plate reader (ThermoFisher).

24 hours after PBMC co-culture killing assay, PBMCs were washed off. ATP assay was performed using CellTiter-Glo 2.0 Cell Viability Assay (Promega) to assess cellular activity of remaining HEK293T cells. Luminescence quantification was measured using Tecan Infinite M1000 Pro plate reader (ThermoFisher).

2.8 Image acquisition

HEK293T cells transfected with TRPV1 were heated to study calcium signaling and NFAT translocation. HEK293T cells were transiently co-transfected with plasmids containing NFAT-EGFP (NFAT linked to EGFP) and R-Geco1 (calcium indicator). 6 hours after transfection, cells were passed to glass-bottom dish pretreated with fibronectin and imaged with Eclipse Ti inverted microscope (Nikon) while being heated using Instec heating stage. Images were captured every 5 seconds for 20 minutes.

2.9 Flow cytometry measurements

The day before drug and heat activation, HEK293T cell lines were seeded into 24-well plates at 30% confluency. 6 hours after drug treatment and heat activation under different conditions, media was aspirated, and cells were trypsinized using TrypLE Express Enzyme (ThermoFisher), washed, and resuspended in 300 ul of PBS. EGFP fluorescent signals were captured using BD Accuri C6 Flow Cytometer (BD Bioscience). Flow cytometry data were analyzed using FlowJo Software.

3. Results

3.1 System design and mechanisms

Our system integrates TRPV1 thermal activation, calcium-calmodulin-calcineurin signaling pathway, and the Tetracycline-On system. As shown in Figure 3.1.1 a, calcium influx as a result of TRPV1 activation by heat or capsaicin will activate components in the signaling pathway and eventually lead to NFAT translocation into the nucleus and activates transcription.

For concept demonstration using luciferase reporters, we developed an AND-gate system under the control of both doxycycline and calcium. As shown in Figure 3.1.1 b, rtTA is fused to the truncated version of NFAT, m1NFAT, which contains only the regulatory domain of NFAT (4 to 399th amino acids) but not the DNA binding domain and used as a shuttling motif in our system. In addition, calcineurin docking sequence of original NFAT1 (PRIEIT, Kd:25μM) was replaced with VIVIT (Kd: 0.5 μM) which has higher affinity to calcineurin. The rtTA-m1NFAT fusion protein is constitutively expressed under a PGK promoter. In the presence of heat, calcium influx through TRPV1 will induce m1NFAT translocation, but the truncated mutant cannot bind to the promoter region to initiate transcriptional activation. Further, in the presence of doxycycline, rtTA will bind to the tetO region (Figure 3.1.1 c) and initiate Firefly luciferase (Fluc) expression. Renilla luciferase (Rluc) is constitutively expressed under a PGK promoter. Similarly, for inducible CD19-EGFP expression, we also developed an AND-gate system under the control of both doxycycline and calcium (Figure 3.1.1 d, e). As a result, a high level of GOI expression is only expected when cells are treated with both doxycycline and TRPV1 activators. The genetic circuit of TRPV1 used for transfection is shown in Figure 3.1.1 f and g.

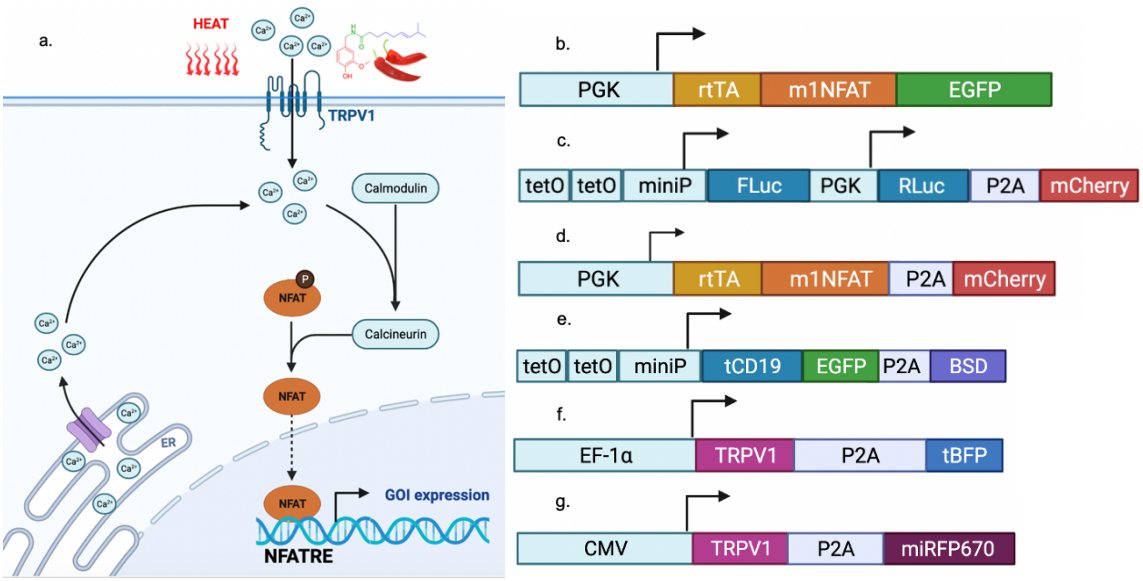

Figure 3.1.1: System design and mechanisms. (a) Calcium signaling and NFAT translocation in response to TRPV1 activation. (b) rtTA and m1NFAT fusion protein regulator genetic circuit. (c) Inducible Firefly luciferase expression under Tet operator control. Constitutive Renilla luciferase used for normalization. (d) rtTA and m1NFAT fusion protein regulator genetic circuit. (e) Inducible tCD19 expression under Tet operator control. (f)(g) TRPV1 plasmids used for transfection and making lentivirus.

3.2 Thermo-activation of TRPV1 and NFAT translocation

We first confirmed that the TRPV1 channel can be activated upon heat. HEK293T cells transiently transfected with TRPV1, NFAT-EGFP, and R-Geco1 (calcium indicator) were imaged under an epi-fluorescence microscope while being heated using a heating platform. Upon heating, we observed a significant increase in calcium signaling in TRPV1-positive cells (Figure 3.2.1.a.i, ii). Fluorescent-labeled NFAT translocation from cytoplasm to nucleus was also observed upon thermal activation (Figure 3.2.1.b.i, ii), and this process was reversible once the heating platform was switched off (Figure 3.2.1.b.iii). We also found the calcium influx and NFAT translocation to be repeatable (Figure 3.2.1.b.iv). Neither NFAT translocation nor increase in calcium signaling was observed in HEK293T cells with low endogenous TRPV1 (Figure 3.2.1.a.iii, iv). These results validate the thermal activation of TRPV1 channels and indicate the potential of using heat-induced

calcium influx to trigger NFAT translocation and consequent GOI expression in TRPV1-positive cells.

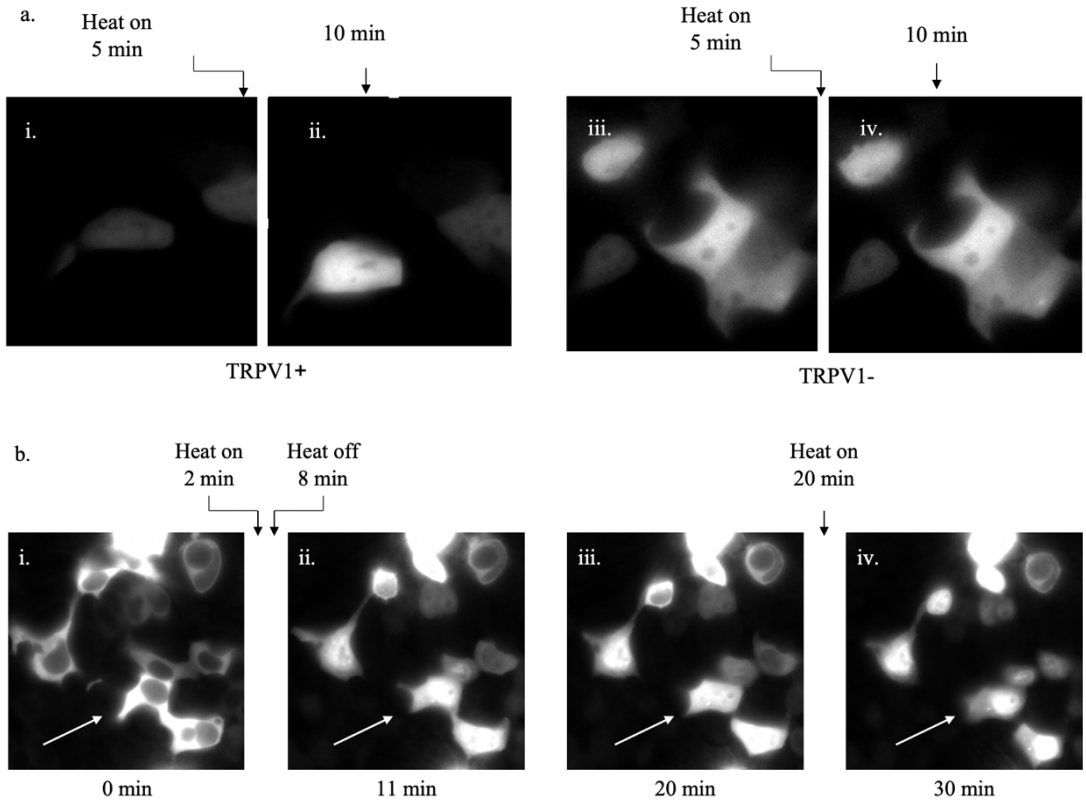

Figure 3.2.1: Calcium signaling and NFAT translocation in HEK293T cells. (a) Calcium signaling after thermal activation. (i, ii) Calcium signaling increased in TRPV1+ cells after thermal activation at 5 min. (iii, iv) There was no significant change in calcium signaling level in TRPV1- cells. (b) NFAT translocation in TRPV1+ cells after thermal activation. (i) NFAT localized in the cytoplasm before thermal activation. (ii) NFAT translocated into the nucleus after thermal activation at 2 min. (iii) NFAT translocated back to the cytoplasm after the heating stage was switched off. (iv) NFAT translocated into the nucleus again after repeated thermal activation at 20 min.

3.3 Luciferase reporter inducibility in HEK293T cells upon thermo-activation

Following visualizing calcium signaling and NFAT translocation in TRPV1-expressing HEK293T cells upon heating, we were interested in whether the signaling cascade can drive GOI expression. We developed a HEK293T cell line expressing rtTA-m1NFAT and TRE-Fluc-PGK-Rluc (Figure 3.1.1.b,c) to demonstrate GOI inducibility using inducible Firefly luciferase and

constitutive Renilla luciferase reporters. We first tested the drug inducibility of the synthetic circuits and measure luminescence at different time points after the drug treatment. As shown in Figure 3.3.1.a, measurement at 6 hours showed the highest normalized luminescence reading for cells treated with both doxycycline and ionomycin. The contrast between doxycycline only and doxycycline and heat double-positive group is also high (11-fold). Thus, we continue to use this 6-hour window in the following experiments. Ionomycin is a membrane permeable ionophore which facilitates cellular calcium ion transport and used as part of our positive control (reference). We found that only when cells were treated with both doxycycline and ionomycin, the normalized luciferase luminescence increased significantly (Figure 3.3.1.b). The data validated the efficiency of our AND-gate system and proved that it is tightly controlled. We then continued to examine GOI expression facilitated by TRPV1 activation-induced calcium signaling.

The HEK293T cell line was transiently transfected with TRPV1 channels, treated with heat and drugs for luminescent reporter gene expression, and analyzed with luciferase assay. As shown in Figure 3.3.1.c, the heat and doxycycline treated group showed significant increase (11.8-fold) in normalized luminescence compared to doxycycline only treated group, showing that heat can activate the TRPV1 channel and induce gene expression, together with doxycycline administration.

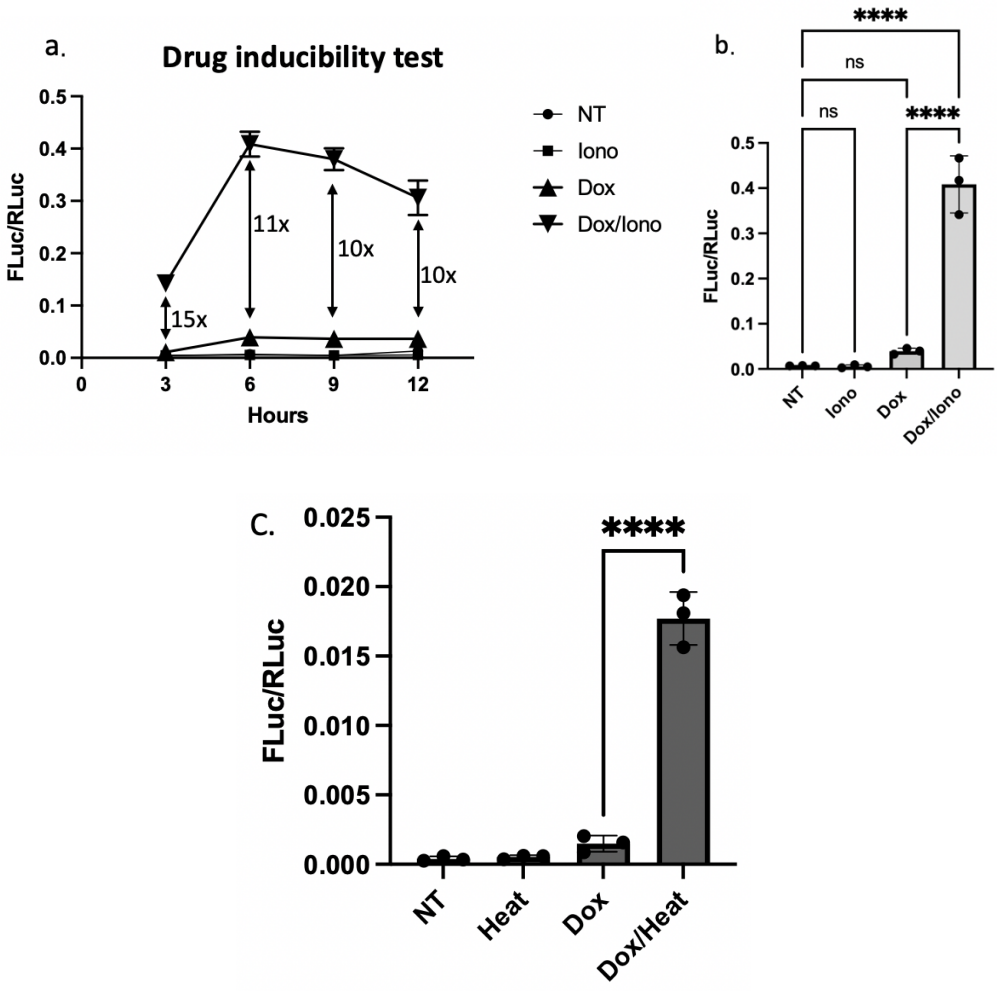

Figure 3.3.1: Characterization of calcium-dependent gene circuit in HEK293T cells. Fluc/Rluc luminescence of HEK293T cells upon (a) drug inducibility test measured at 3, 6, 9, 12 hours. (b) drug inducibility test measured at 6 hours. (c) heat and drug treatment. (a) Measurement after 6 hours showed the highest Fluc/Rluc normalized reading for the double-positive group and a big contrast between doxycycline only and doxycycline and heat. (b) The result is only statistically significant for the double-positive group, indicating the efficiency of our system. (c) TRPV1+ cells showed 11.8-fold increase in normalized luminescence compared to doxycycline only treated group. Statistical significance by Tukey's test is indicated by asterisks (**** $P \leq 0.0001$).

3.4 Luciferase reporter inducibility in Jurkat T cells upon thermo-activation

As discussed in chapter 1.4, we want to 1) either control CAR expression in T cells such that the CARs are only expressed when they are in the correct tumor environment, or 2) specifically

engineer tumor cells with target antigens such that only the engineered cells are recognized by CAR-T cells and killed.

For the first strategy – application on T cells, since it was reported that endogenous TRPV1 expression level is relatively high on T cell plasma membranes, we envisioned heat-induced calcium influx through endogenous TRPV1 could be sufficient to facilitate NFAT translocation driving CAR expression. As such, we can utilize this inducible system and turn T cell into CAR-T cell when it is in the tumor environment through applying heat locally.

We started off by examining the change of calcium signaling in T cells when they are heated. We heated Jurkat T cells and stained cells with Fluo-4 AM (a green calcium indicator). As shown in Figure 3.4.1, calcium intensity in Jurkat T cells increased 1.77 folds after heating. Although this change was not very significant, we proceeded with using endogenous TRPV1 in Jurkat T cells to see if we can achieve a detectable level of change in GOI expression.

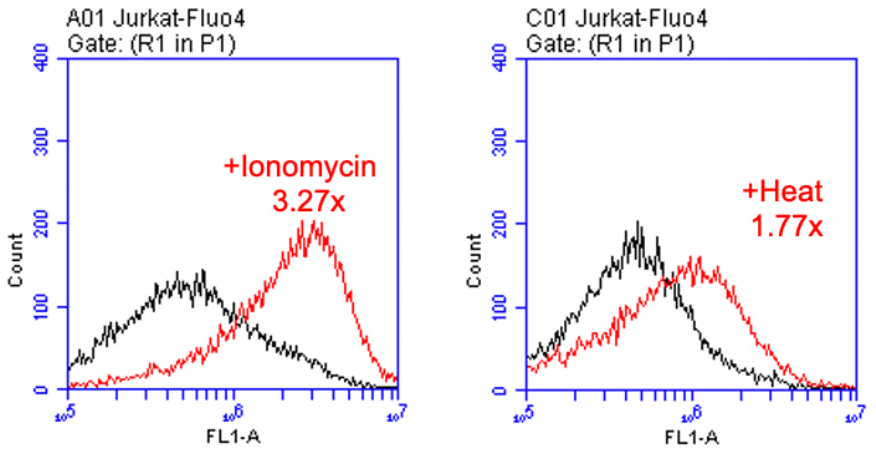

Figure 3.4.1: Calcium signaling in Jurkat T cells. After heating, calcium intensity in Jurkat T cells increased 1.77 folds. Positive control (cells treated with ionomycin) showed 3.27 folds of increase.

We proceeded with demonstrating induced luciferase reporter gene expression in Jurkat T cell. We developed a Jurkat T cell line stably expressing the same rtTA-m1NFAT and TRE-Fluc-

PGK-Rluc. The Jurkat cells were thermally activated in water bath and treated with doxycycline for luminescent reporter gene expression, followed by luciferase assay. Unfortunately, as shown in Figure 3.4.2, there was no statistically significant difference in luminescence between doxycycline only treated group and doxycycline and heat-treated group. These data suggested that the expression level of endogenous TRPV1 in Jurkat T cells was not sufficient of activating GOI expression.

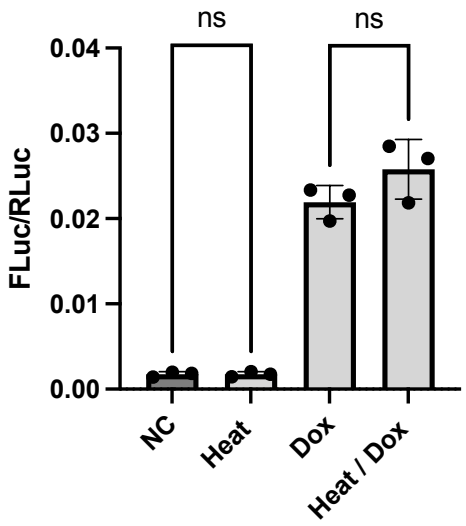

Figure 3.4.2: Characterization of calcium-dependent genetic circuit in Jurkat T cell. Fluc/Rluc luminescence of Jurkat T cells upon thermal activation and drug treatment in water bath was measured. No significant luminescence differences were observed for negative control vs heat, doxycycline vs doxycycline only and heat and doxycycline. Tukey's test was performed to examine statistical significance of the data.

In order to overexpress TRPV1 in Jurkat T cell membrane, we transiently transfected plasmids containing TRPV1 sequence into the Jurkat T cell line by electroporation. On the following day we examined the transfection efficiency to be about 50%, and cells were thermally activated and treated with doxycycline, followed by luciferase assay. Unfortunately, normalized luminescence from doxycycline and heat-treated group did not differ statistically from doxycycline only group (both 6.7-fold, Figure 3.4.3).

We suspected that T cells regulate their endogenous TRPV1 expression level tightly and TRPV1 is only elevated during T-cell activation [38]. We then created a T cell line stably expressing the same rtTA-m1NFAT and TRE-Fluc-PGK-Rluc with PBMCs. The T cells were activated with Dynabeads, thermally activated, and treated with doxycycline for luminescent reporter gene expression. However, we still did not see significantly different luminescence between doxycycline group and doxycycline and heat group.

After examining the data obtained so far, we decided to shift gear from targeting T cells to investigating tumor cells' response as described in aim 2.

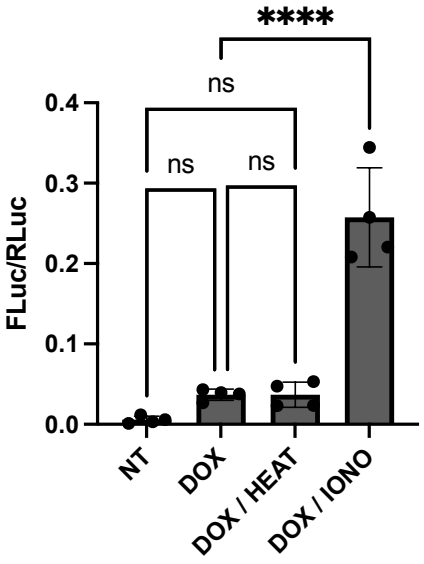

Figure 3.4.3: Characterization of calcium-dependent gene circuit in TRPV1+ Jurkat T cell. Fluc/Rluc luminescence of Jurkat T cells transfected with TRPV1 was measured upon thermal activation and drug treatment. Doxycycline treated group and doxycycline and heat-treated group both showed 6.7-fold increase compared to the negative control. In contrast, positive control-doxycycline and ionomycin treated group showed a 46.9-fold increase in luminescence reading. Tukey's test was performed to examine statistical significance of the data.

3.5 CD19 inducibility in HEK293T cells upon thermal activation

Since both endogenous and overexpressed TRPV1 in Jurkat T cells did not demonstrate the potential for significant reporter gene expression, we moved forward with the 2nd strategy:

integrating TRPV1 channels into tumor cells. We want to specifically engineer tumor cells with inducible target antigens expression such that only the engineered cells are recognized by CAR-T cells and killed.

In tumor cells, we envision that heat-induced calcium influx through TRPV1 could facilitate NFAT translocation and induce target antigen expression on cell membrane. Since we successfully demonstrated TRPV1 thermo-activation and GOI expression with the HEK293T cell luciferase reporter model, we created a HEK293T cell line stably expressing rtTA-m1NFAT and TRE-tCD19-EGFP to demonstrate induced CD19 expression upon TRPV1 thermal activation and drug treatment (Figure 3.1.1 d, e).

We first tested the drug inducibility of the rtTA-m1NFAT/TRE-tCD19-EGFP HEK293T cells. A shown in Figure 3.5.1.a.iv, engineered HEK293T cells showed 38.4% of CD19-EGFP inducibility when treated with both doxycycline and ionomycin, and relatively low leakages (7.43%). Cells treated with either doxycycline or ionomycin did not show much induction.

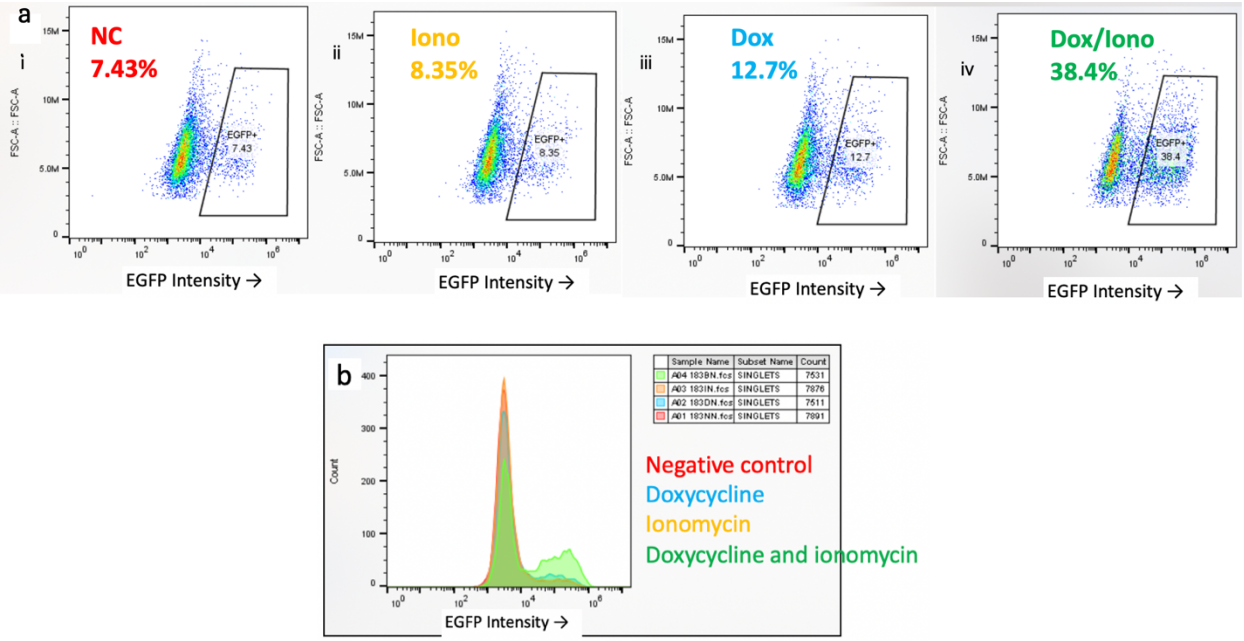

Figure 3.5.1: Characterization of inducible tCD19 in HEK293T cells. (a)Flow cytometry plots of CD19-EGFP drug inducibility in HEK293T cells were shown. Engineered cells were treated with (i) Negative control. (ii) Ionomycin only. (iii) Doxycycline only. (iv) Both Ionomycin and doxycycline. (b) Histogram of EGFP intensity of the engineered cells under different treatments.

The rtTA-m1NFAT/TRE-tCD19-EGFP HEK293T cells were then transiently transfected with TRPV1 channels, treated with heat and drugs for CD19-EGFP expression, and the CD19-EGFP expression level is analyzed using flow cytometry. As shown in Figure 3.5.2.a.iv, TRPV1-positive cells showed 31.3% induced CD19-EGFP when treated with doxycycline and activated thermally, whereas other treatment groups (Figure 3.5.2.a.i, ii, iii) did not show much CD19-EGFP induction. Figure 3.5.2.b showed the same results in clearer pictures with histograms. The difference in percentage of cells induced with CD19-EGFP is significant between doxycycline only treated group and doxycycline and heat-treated group (Figure 3.5.2.c). The above data all showed the inducibility of the target antigen when given both thermal activation and drug in HEK293T cells with our system.

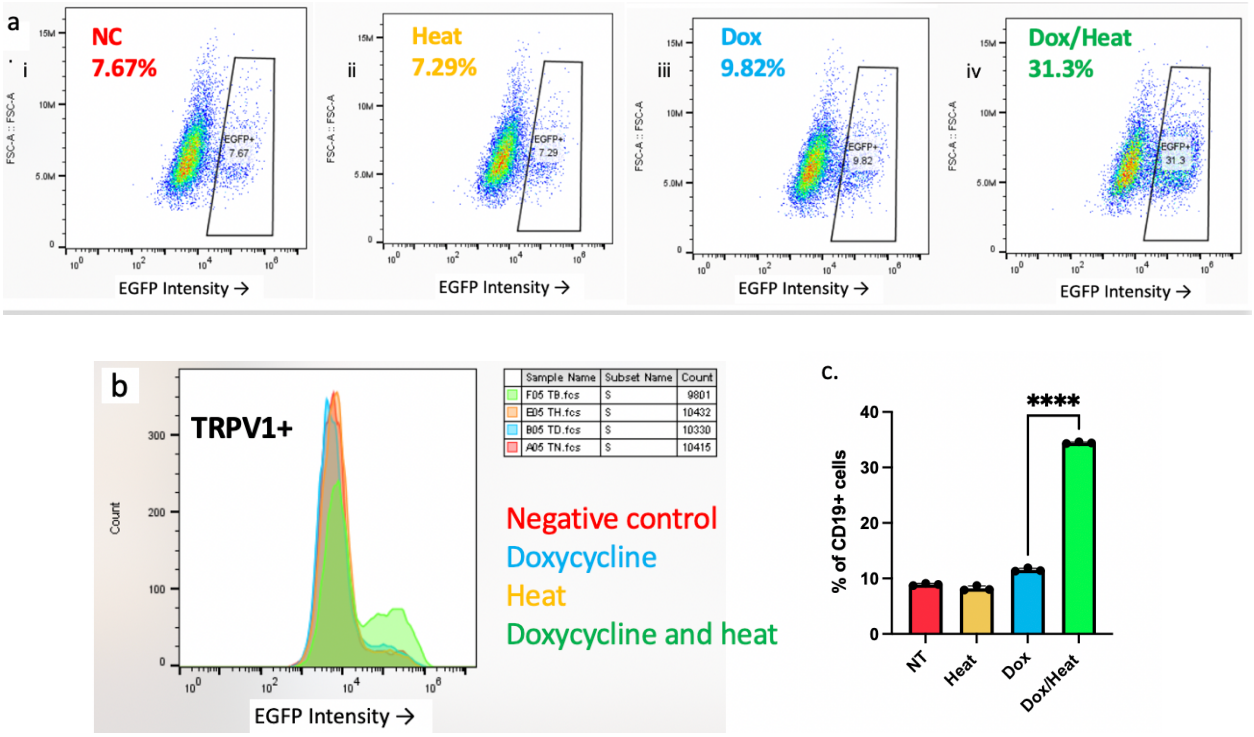

Figure 3.5.2: Characterization of inducible tCD19 in TRPV1+ HEK293T cells. (a) Flow plots of CD19-EGFP inducibility in engineered HEK293T cells upon thermal activation and drug treatment. (i) Negative control. (ii) Heat only. (iii) Doxycycline only. (iv) Both heat and doxycycline. (b) Histogram of tCD19 inducibility in engineered HEK293T cell line upon thermal activation and drug treatment. (c) Statistics of triplicate of tCD19 inducibility in engineered HEK293T cell line upon thermal activation and drug treatment. Statistical significance by Tukey's test is indicated by asterisks (****: $P \leq 0.0001$).

3.6 Killing assay: co-culture with anti-CD19 CAR-T cells

We successfully demonstrated target antigen CD19 inducibility in thermally activated and drug treated rtTA-m1NFAT/TRE-tCD19-EGFP HEK293T cells. We then co-cultured these engineered HEK293T cells together with anti-CD19 CAR-T cells to demonstrate targeted killing of HEK293T cells presenting induced antigens. Theoretically, induced HEK293T cells will present CD19 antigen on their cell surface upon heat and doxycycline treatment, and anti-CD19 CAR-T cells will recognize the antigen and attack on HEK293T cell.

The engineered HEK293T cells were transiently transfected with TRPV1 channels, treated with heat and drug for CD19-EGFP expression, then co-cultured with anti-CD19 CAR-T cells. We

measured cell viability of the remaining cells 24 hours after the killing assay using ATP luciferase assay, normalized to freely growing mono cultured engineered HEK293T cells with treatment. A lower cell viability directly correlates to stronger killing by anti-CD19 CAR-T cells. Doxycycline and heat-treated group showed 67.4% decrease of cell viability compared to negative control group. Other groups did not show significant decrease of cell viability. As such, we demonstrated the feasibility of using thermal activation and doxycycline to induce target antigen in tumor cells, making them recognizable and attackable by anti-CD19 CAR-T cells.

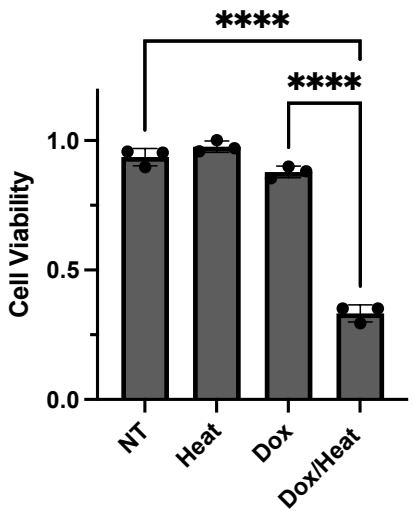

Figure 3.6.1: Anti-CD19 CAR-T cell killing assay. ATP luminescence of HEK293T cell line transfected with TRPV1 upon thermal activation and drug treatment was measured. Doxycycline and heat-treated group showed 67.4% decrease of cell viability compared to negative control group. Tukey's test was performed to examine statistical significance of data. Statistical significance is indicated by asterisks (****$P \leq 0.0001$).

We also conducted control experiments for the anti-CD19 CAR-T cell co-culture assay. We co-cultured plain HEK293T cells, our engineered cell line (rtTA-m1NFAT/TRE-tCD19-EGFP HEK293T cells) and our engineered cell line transfected with TRPV1 together with anti-CD19 CAR-T cells. After 24 hours we examined the viability of the remaining cells using ATP luminescence assay. We found that plain HEK293T cells and the engineered HEK293T cell line were not killed by the CAR-T cells, no matter they were not treated or treated with both heat and

doxycycline (Figure 3.6.2). For the engineered HEK293T cell line transfected with TRPV1, cells were not killed by anti-CD19 CAR-T cells when they were not treated, but significantly killed (with only 32.6% of cells remained) after 24-hour co-culture. The control experiment showed that the genetic circuit system we designed is precisely and tightly regulated by heat and doxycycline. we can thus apply this system to any tumor cell type and direct them for corresponding CAR-T cell attack.

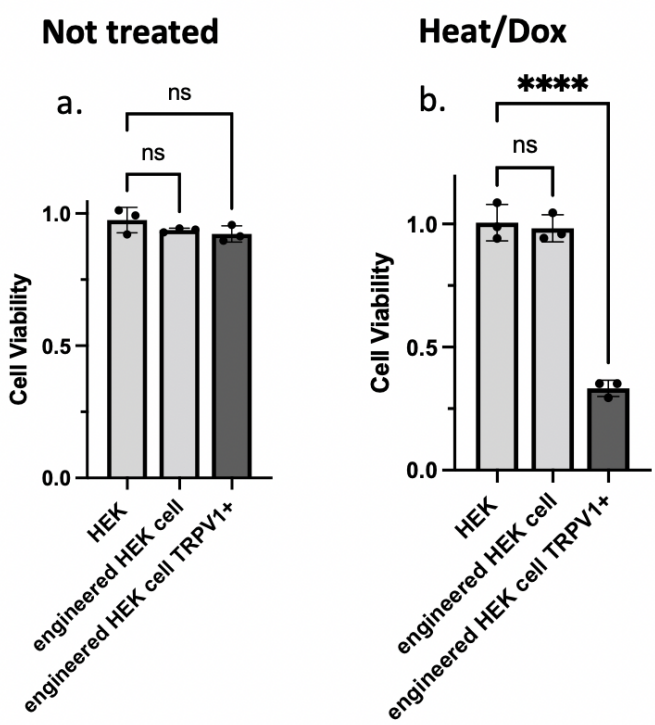

Figure 3.6.2: Anti-CD19 CAR-T cell killing assay control experiments. (a) Cell viability 24 hours after co-culturing plain HEK293T cells, rtTA-m1NFAT/TRE-tCD19-EGFP HEK293T cells and rtTA-m1NFAT/TRE-tCD19-EGFP HEK293T cells transfected TRPV1 with anti-CD19 CAR-T cells, not treated. Cells were not killed. (b) Cell viability 24 hours after co-culturing plain HEK293T cells, rtTA-m1NFAT/TRE-tCD19-EGFP HEK293T cells and rtTA-m1NFAT/TRE-tCD19-EGFP HEK293T cells transfected TRPV1 with anti-CD19 CAR-T cells, treated with doxycycline and heat. Only rtTA-m1NFAT/TRE-tCD19-EGFP HEK293T cells transfected TRPV1 were significantly killed (with only 32.6% of cells remained) after 24-hour co-culture. Statistical significance by Tukey's test is indicated by asterisks (****$P \leq 0.0001$).

3.7 Cell viability analysis

In chapter 3.6, we demonstrated the feasibility of using thermal activation and doxycycline to induce target antigen expression in tumor cells, which direct them for CAR-T cell recognition and attack. At the same time, we are aware of the fact that noxious heat may kill cells in an undesirable manner. We then examined the effect of our pulsed heating pattern on cell viability, and also included another commonly used heating pattern (12 minutes continuous heating at 43 °C) for initiating heat shock promoter (Hsp) activation as a reference.

As shown in Figure 3.7.1, our heating pattern which can sufficiently activate TRPV1 channels (pulsed heating: 43°C for 2 minutes, cool down at 37°C for 3 minutes, cycle repeated 6 times) only showed 7.4% decrease of viability, whereas continuous heating for 12 minutes led to 15.9% decrease of viability. Channel-like response is fast, so TRPV1 activation can be achieved with pulsed heating for shorter duration of time. As a result, cell viability after heating is less compromised comparing to traditionally used continuous heating pattern for Hsp.

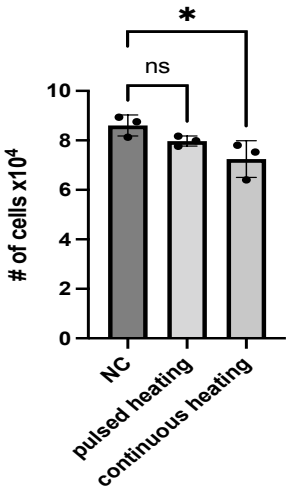

Figure 3.7.1: Cell viability assay after heat shock. Number of remaining cells was measured 6 hours after heat shock in water bath. NC: negative control; Pulsed heating: TRPV1 heating pattern; Continuous heating: 12 minutes continuous heating. Statistical significance by Tukey's test is indicated by asterisks (*: $P \leq 0.05$).

3.8 Repeatability of TRPV1 thermal activation

We also tested the potential of repeated TRPV1 thermal activation. We observed that further activating the TRPV1 channels by heating HEK293T cells and administering capsaicin (a TRPV1 activator) can induce different level of GOI expression. As shown in Figure 3.8.1.a, capsaicin, doxycycline and heat-treated group showed higher normalized luminescence compared to doxycycline and heat-treated group. We then move on to examine the effect of different heating pattern on GOI expression level using luciferase reporter.

We first heated doxycycline treated engineered HEK293T cells for 30 seconds in a thermocycler, with 4 minutes and 30 seconds cool down time after each heat shock. As shown in Figure 3.8.1.b, cells heated 4 times showed 3.4-fold higher normalized luminescence as compared to doxycycline only treated group; cells heated more times showed even higher fold change (5.9-fold for 6 times heating and 7.2-fold for 8 times heating). This result demonstrated that the TRPV1 channel can be heated repeatedly to achieve different levels of GOI expression.

Furthermore, we also varied the duration of each heat shock. We heated doxycycline treated engineered HEK293T cells for 10 seconds (4 minutes and 50 seconds cool down), 20 seconds (4 minutes and 40 seconds cool down), 30 seconds (4 minutes and 30 seconds cool down) and 60 seconds (4 minutes cool down) for 6 times. As shown in Figure 3.8.1.c, 6-time heating for 10s already showed 3.7-fold increase in normalized luminescence compared to doxycycline-treated group. 6-time heating for 20s and 30s showed 5.3-fold and 7.2-fold increase in normalized luminescence respectively. However, 6-time heating for 60s showed decrease in fold change when compared to 6-time heating for 30s. This might be due to apoptosis caused by calcium overdose resulted from over-activating TRPV1 channels.

Overall, the TRPV1 channels can be heated repeatedly, to achieve different level of GOI expression meeting needs for various applications.

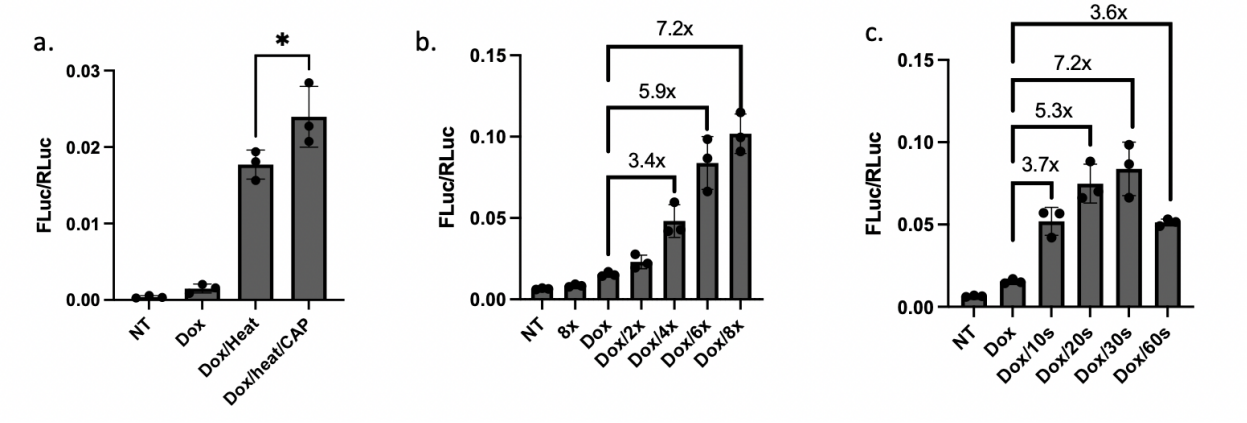

Figure 3.8.1: Repeatability of TRPV1 thermal activation. (a) Feasibility of heating cells for a longer period to achieve higher GOI expression. Capsaicin, doxycycline, and heat-treated group showed higher normalized luminescence compared to doxycycline and heat-treated group. (b) Normalized luminescence when cells were heated in thermocycler for 30 seconds for different cycles. Normalized luminescence increases when cells were heated for more cycles. (c) Normalized luminescence when cells were heated in thermocycler for different duration for 6 cycles. Statistical significance by Tukey's test is indicated by asterisks (*: $P \leq 0.05$).

4. Discussion

Although CAR-T cell therapy has revolutionized cancer treatment, on-target off-tumor toxicity remains a significant risk hindering its broader applications. The current novel CAR designs and applications still highlight the need for a tightly regulated inducible system with high spatiotemporal control.

In the system we designed, we used TRPV1 thermal activation-induced calcium signaling driving NFAT translocation to initiate GOI transcription (Figure 3.2.1). However, because of the ubiquitous presence of calcium ion and the dynamicity of calcium signaling, it is best used when paired with another level of regulation, to prevent basal transcriptional leakage and achieve tighter control of gene expression. As a result, we incorporated the Tet-On system in our system design, and the AND-gate genetic circuits is only activated when both calcium and doxycycline are present (Figure 3.1.1).

The AND-gate genetic circuit allows precise spatiotemporal control of GOI expression via localized, targeted heating and drug administration at fixed timing. We successfully demonstrated luciferase reporter gene Firefly luciferase expression in HEK293T cells overexpressing TRPV1 when they were thermally activated and treated with doxycycline (Figure 3.3.1). This result laid the foundation for the project and indicated the feasibility of using TRPV1 to upregulate calcium signaling, and consequently gene expression.

Following that, we substituted the luciferase reporter genes with CD19, a target antigen. We also successfully induced CD19 expression for TRPV1 positive HEK293T cells when they were thermally activated and treated with doxycycline (Figure 3.5.2). Upon co-culture with anti-CD19 CAR-T cells, a large population of heat and doxycycline treated cells were recognized and killed by anti-CD19 CAR-T cells (Figure 3.6.1). Overall, we demonstrated the feasibility of using

thermal activation and doxycycline to induce target antigen expression in tumor cells, which direct them for CAR-T cell attack.

Each component of our system is highly modular. We can easily switch the target antigen and CAR pair to aim at different tumor types. In addition, the rtTA, Tet operator, and other molecular components will continue to evolve to achieve greater precision for higher spatiotemporal control with drug and heat.

Doxycycline is also served as a safety measure in the system. Sometimes cells may be under heat stress, or there are increased calcium signaling due to other process; circuits that can be activated by calcium alone may suffer from high basal gene expression leakages. Using doxycycline as a layer of control of the genetic AND-gate will help prevent undesirable GOI expression. We can also achieve different GOI expression level by altering doxycycline concentrations.

For *in vivo* studies and real applications, we plan to induce temperature changes at confined volume via focused ultrasound, which has the potential to target human tissues safely and noninvasively with high spatial resolution [39]. The oscillating pressure and mechanical loading and unloading of focused ultrasound can result in localized heat generation at confined volume, inducing temperature increase and TRPV1 channel opening [40].

Focused ultrasound allows us to reach tumor tissues that are tens of centimeters under the skin, making itself superior to optogenetics, which is always associated with the problem of limited penetration depth due to light scattering [39]. Previous study has demonstrated the feasibility of using short-pulsed ultrasound to thermally activate CAR-T cells under heat shock promoter (Hsp) regulations [40]. However, Hsp activation requires continuous heating (usually 10-15 minutes) for transcriptional initiation, which may result in reduced viability of cells subjected to focused

ultrasound. In contrast, channel-like response is fast, so TRPV1 activation can be achieved with pulsed heating for as short as 10 seconds (Figure 3.8.1). As a result, cell viability after heating is less compromised comparing to the system with Hsp (Figure 3.7.1). In addition, being a channel, TRPV1 can be heated repeatedly while maintaining a high intensity of calcium signaling (Figure 3.2.1), to achieve different levels of GOI expression (Figure 3.8.1). We can thus activate the channel for longer period and provide longer activation period for GOI expression.

In practice, our technology could be realized with gene therapy technologies [41]. The synthetic genetic circuits could be delivered into tumor cells *in vivo* through adeno-associated virus (AAV). As a result, once given focused thermo-activation and doxycycline, tumor cells will present the specific target antigen on their surface and become recognized by CAR-T cells. This method causes low immunogenicity because no genome integration is associated in the AAV infection process [42]. In addition, for real application, we plan to deliver CAR-T cells to tumor sites with local injection. This method also helps to solve the problem of T cell homing failure associated with intravenous delivery [43].

Unfortunately, heat and drug inducibility test on Jurkat T cells suggests their limited expression of TRPV1 membrane proteins (Figure 3.4.1, Figure 3.4.2), which corresponds to previously reported TRPV1 gene expression data of Jurkat T cells [44]. We then proceeded with overexpressing TRPV1 channels in Jurkat T cells but didn't achieve desirable results (Figure 3.4.3). We found out that T cells have precisely regulated TRPV1 level during different maturation and development stages, and it may be difficult to control TRPV1 expression levels in T cells by simply transfecting or transducing the TRPV1 gene [45]. Also considering the fact that there might be intrinsic differences between Jurkat T cells and PBMCs, PBMCs may respond differently from Jurkat cells and give more flexibility in TRPV1 expression level. In the future, we may consider

exploring T cell TRPV1 thermal activation potential at different T cell differentiation and activation stages.

5. Conclusions and Future Work

This project demonstrated the feasibility of engineering cells to respond to thermal induction and, in turn, express genes of interest via synthetic circuits. We first demonstrated the concept with luciferase reporters in HEK293T cells and then successfully incorporated similar genetic circuits into HEK293T cells to present CD19 target antigens which are recognized by anti-CD19 CAR-T cells for temperature-controlled activation.

In the future, we can try to overexpress human version of TRPV1 by transduction in tumor cells such as MDA-MB-231 and Nalm-6, and screens for the thermal activation potential of the channel in various tumor cell types. An appropriate approach is to start with cells that have relatively higher endogenous TRPV1 expression level and further overexpress TRPV1 in these cells using a titratable amount of TRPV1 virus.

Furthermore, focused ultrasound, with high spatiotemporal resolution, can deliver energy and generate heat to centimeters below skin. We envision in the future with a well-established focused ultrasound system in the Wang Lab, we can induce temperature changes in a confined volume of deep tissue, and together with the described system in this thesis, we will be able to address the concerns of on-target off-tumor toxicities associated with current CAR-T therapies. I hope that my thesis will provide a fundamental groundwork for this rather innovative therapy.

Material in this thesis is co-authored with Dr. Chi Woo Yoon. The thesis author was the primary author of this material.

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