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Next generation armored CAR-T cells with a drug inducible cytokine circuit

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Abstract. Immunotherapies which direct the body's own immune system against tumors are a safer and more efficacious alternative to other types of cancer treatments. Chimeric Antigen Receptor or CAR T cell therapy has emerged at the forefront of contemporary cancer immunotherapy research and is highly specific for a wide range of cancer types, resulting in improved patient outcomes. Early generations of CAR T cells were ineffective and failed to proliferate due to lack of costimulatory signals and immunosuppression within the tumor microenvironment (TME), but later generations of "armored" CAR T cells added costimulatory receptor domains and constitutive expression of stimulatory immunocytokines to ameliorate this. However, increased aggressiveness in T cells comes with issues such as off-site toxicity, fails to address relapse due to immune evasion, and combined with the prohibitive cost of engineering CAR T cells limit the efficacy of the treatment. Next generation CAR T-cells address these problems with the engineering of synthetic biological circuits that provide selective control over immune function in response to inducers. In this research proposal, we design a next generation armored CAR T cell with a small molecule inducible cytokine circuit, combining different synthetic biology approaches from previous research on CAR T cells to enhance the safety of the therapy by providing a reversible, safe, and rapid method of modulating CAR T cell stimulation.

Keywords: Armored CAR T-Cells, synNotch Receptor, Synthetic Cytokine Circuit, Research Proposal

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1. Introduction

Cancer is a significant health issue causing over 10 million deaths annually, with lung, colorectal, liver, stomach and breast cancers making up the most significant sources of mortality [1]. Traditional treatment options such as radiotherapy, chemotherapy, and surgery are limited, with severe side effects, and patients with metastatic and recurrent cancers face an abysmal prognosis [2]. Research into alternatives has focused on precision therapies using medicine that reduces toxic effects on the body [3]. One such treatment in this class is immunotherapy which enhances the natural ability of the immune system to recognize and destroy tumor cells [3]. Early attempts focused on adoptive T-cell therapy, where T cells that can recognize specific tumor antigens are extracted and grown from the patient [3]. However, this method is limited to resectable tumors with tumor-infiltrating lymphocytes present [3]. In order to increase the potency and specificity, TCRs with known tumor antigens are inserted, but this can result in immune rejection due to incompatibility with the host's MHC (major histocompatibility complex) molecules, which mediate self-foreign immune recognition [3].

To address this, Chimeric Antigen Receptor (CAR) T cells engineered synthetic receptors consisting of a single chain variable fragment, a fusion protein consisting of light and heavy chain variable regions of an antibody connected by a linker region, linked to a TCR signaling domain, were developed to bypass the MHC by directly binding to antigens and activating T cell signaling [3,4]. In contrast to conventional treatments, CAR T cell therapies are less aggressive, more specific to tumors, and involve just a single infusion and less than two weeks of hospitalization, improving patients' overall quality of life [3]. Overall, CAR T cells have been one of the most significant developments in anti-tumor immunotherapy over the past decade.

Several CAR T cell generations have been successfully developed. First-generation CAR- T cells show antigen-specific toxicity to cancer cells but require a costimulatory signal like natural T cells to be fully activated [3]. Without this T cells can become exhausted (anergy), have limited persistence, and little therapeutic effect [3]. Second-generation CARs include a costimulatory domain and third-generation CARs include a second costimulatory domain to further improve T-cell persistence and activation [3]. While these early-generation CAR T cells show great clinical benefit to patients suffering from hematological cancers [2,3], the same success has not been shown for patients suffering from solid tumors [2]. Various interactions between immune cells mediated by cytokines (signaling molecules such as interleukins, or ILs), chemokines, and growth factors occur within a solid tumor mass, making up the tumor microenvironment (TME), and some of these interactions can result in immune suppression of tumor infiltrating lymphocytes [2]. 4th generation "armored" CAR T cells engineered with various accessory molecules to overcome the immunosuppressive TME are currently being developed [2,3]. However, a significant obstacle is the overstimulation of the host immune system through excessive cytokine secretion causing cytokine release syndrome, marked by systemic inflammation, hypotensive shock, and multiple organ failure [2,3]. Early methods for dealing with cytokine release syndrome rely on neutralizing cytokines with antibodies or building a kill switch to destroy CAR T cells, resulting in cancer relapse and increased patient mortality [2]. Cancer cells can also evade the immune system through mutations altering their surface antigens, a process called antigen escape [3]. CAR T cells with multiple antigen receptors reduce the chance of this but increase the risk of CAR T cells attacking healthy cells that often express the same antigens as cancer cells, resulting in on-target, off-tumor toxicity [2,5]. Creating new CAR T cells is a time-consuming, labor-intensive, and prohibitively expensive process, making it infeasible to treat patients' multiple times, especially if they are in the advanced stages of cancer [6]. A method to readily modulate CAR T cell function *in vivo* would overcome many of these barriers and is the current focus of research into next-generation CAR T cells. An emerging approach to developing next-generation CAR T cells harnesses the tools of synthetic biology to design artificial biological circuits [4,6,7]. Four studies are selected below to explain the current advances and approaches to developing CAR T cells controlled by inducible circuits.

A study demonstrated a synthetic cytokine circuit that reversibly induce the release of cytokines in an antigen-dependent manner to overcome immune suppression in the TME [8]. The circuit was built on a synthetic Notch receptor (synNotch), a chimeric receptor with high antigen affinity from its single

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chain variable fragment (scFv). When activated synNotch cleaves a transcription factor that upregulates a transgene encoding sIL-2, a "super" cytokine with increased receptor affinity, and allows tumor-localized cell-autonomous cytokine delivery, proliferation, and enhanced cytotoxicity of CAR T cells [8]. This synNotch sensor was tested *In vivo* with grafted human lymphoma cells displaying model antigen CD19 in immunocompromised mice and exhibited improved tumor clearance without off-target toxicity when analyzed by flow cytometry for tumor infiltrating lymphocytes and T cell expansion [8]. Another test with anti-NY-ESO-Q TCR expressing T cells targeting melanomas in NSG mice demonstrated that both autocrine and paracrine cytokine signaling enhanced T cell cytotoxicity. Paracrine signaling, however, was found to be less effective due to inhibition and competition from native IL-2 up taking T cells [8].

Another study explored a different approach to programming CAR T-cells involving directing focused ultrasound pulses (FUS) towards T cells in tumors guided by magnetic resonance imaging (MRI) [7]. FUS targeting localizes CAR T-cell activation within the tumor region, mitigating off-target effects [7]. FUS-activated CAR T-cells contain a heat-shock-activated promoter derived from heat shock protein 90 (Hsp90) followed by a Cre recombinase encoding transgene [7]. The promoter is activated by heat pulses above 40 °C, letting the recombinase excise a STOP sequence further downstream, allowing expression of the CAR receptor [7]. This method keeps the effectiveness of CAR-T cell therapy without systemic toxicity and allows precise and reversible control over T cell activation [7]. This therapy remains in its early stages, with ongoing developments in acoustogenetics promising further advancements [7].

Several recent studies have also explored programmable CAR activation by engineering small molecule inducible circuits [6]. Inserting protease domains subject to inhibition by clinically approved drugs that reversibly degrade CARs allows reversible dosage-dependent control over CAR-T cell activity [6]. Universal CARs utilize adaptor proteins that bridge scFvs with different antigen specificities and CAR signaling domains, allowing CAR antigen specificity to be switched [6]. These systems work with multiple immune cell types and antigen targets and allow the construction of complex combinatorial logic gates beyond ON and OFF switches that fine-tune CAR T-cell function and toxicity [6]. These circuits are versatile, broadly applicable, and demonstrate great therapeutic potential [6].

A dual vector system (usually two separate viruses) is commonly used for CAR T-cell expression to express multiple types of receptors [9]. However, this is a limiting factor due to the expense of cell sorting and low translational potential [9]. Uni-Vect is a genetically integrated system combining constitutive and antigen-inducible effector molecule expression in a single vector [9]. In the first Uni-Vect construct featured in the paper, IL-12 secreting CAR-T cells were made by combining inducible IL-12 transgene with a CAR constitutive gene, eradicating solid tumors *in vivo* without causing lethal systemic toxicity [9]. The second construct ameliorates cytokine release syndrome via iToci, a fusion protein from a human IgG Fc with an IL-6Ra blocking scFv [9]. Lastly, antigen-inducible transcription factor expression reduces the risk of malignant transformation or autoimmunity [9]). The advantage of the Uni-Vect system is in adding new functions to the CAR framework as well as modulating CAR T cell function.

We selected drug-inducible and cytokine circuits as an area of further research. We hypothesize that GZB drug-induced circuits can control cytokine release in CAR T-Cells in a reversible and dosage-dependent manner and are able to reverse the effects of CRS *in vivo*.

2. Experimental Method

The primary component we propose for our cytokine circuit is a drug-gated synNotch ON receptor with four parts– a single chain fragment variable (scFv) antigen recognition domain, an inhibitable non-structural protein 3 (NS3) viral protease domain that cleaves and inactivates the receptor [6], shown in Figure 1, and a synthetic transcription factor signaling domain that upregulates the expression of an IL-2 transgene [8], shown in Figure 2. NS3 is a promising protease system used in a past study and is found in the hepatitis C virus (HCV), where it cleaves the viral protein at specific junction sites [6]. It is inhibited by clinically approved drugs such as grazoprevir (GSV). IL-2 is a proinflammatory cytokine

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[8] that stimulates T cell proliferation and cytotoxicity [5,8]. In the resting state without GSV as an inhibitor, the receptor is inactive as it is cleaved and fragmented by NS3 [6]. In the active state, GSV blocks NS3 from cleaving the receptor, releasing cytokines, and acting as an ON switch for T-cell activation [6].

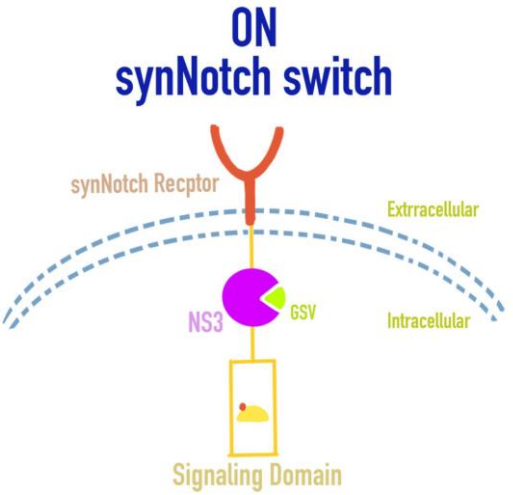

Figure 1. Location of major structural components of the drug gated synthetic Notch (synNotch) switch, with the extracellular single chain variable fragment (scFv) antigen receptor in orange, the transmembrane domain containing hepatitis C virus (HCV) protease nonstructural 3 (NS3) along with grazoprevir (GSV) inhibitor in green, and intracellular signaling domain with transcription factor for IL-2 transgene in yellow [6].

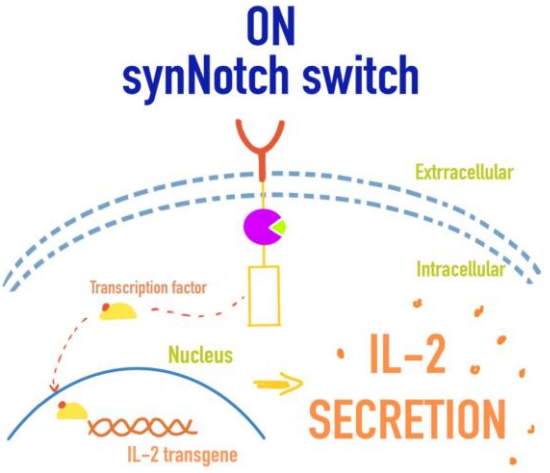

Figure 2. The drug inducible synNotch circuit in full, showing the TF entering the nucleus, binding to the IL-2 transgene promoter, and inducing IL-2 expression [8].

We first focus on testing and fine-tuning this system *in vitro* with CD19+ NALM6 human lymphoma cells, a standard model [6]. Two experimental groups and two control groups will be set up for this test– an anti-CD19 CAR T cell population without the drug-gated anti-CD19 receptor treated with and without GSV as the control groups, and an anti-CD19 CAR T cell population with the anti-CD19 drug-gated receptor treated with and without GSV as the experimental groups (Figure 3).

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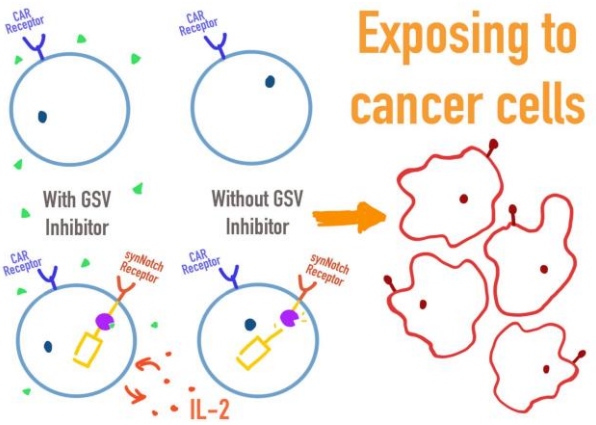

Figure 3. The experimental and control groups of the in vitro test are shown. The experimental group includes CARs with the drug-gated synNotch receptor with and without GSV and the control group includes traditional CAR T cells with and without GSV. These groups are exposed to cancer cells.

In vivo testing will occur with the same groups with NSG mice, which are completely immunodeficient and serve as a host for xenografted human tumors [6], as shown in Figure 5a,b. Some commonly researched solid tumors include colorectal tumors, which exhibit EGFR antigens, and pancreatic tumors, which exhibit HER2 antigens [2]. Taking samples from the site of the tumor to measure the counts of each cell type through flow cytometry (Figure 4c) will indicate how effective the synthetic cytokine circuit is at regulating T cell proliferation.

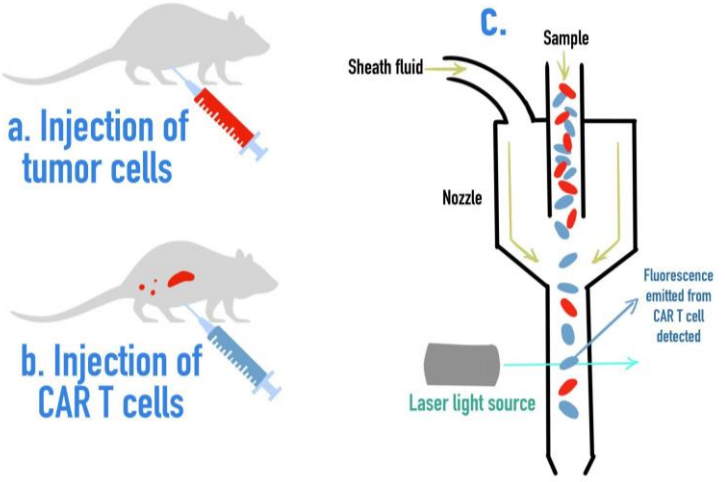

Figure 4. (a) Procedure for the xenografting of human tumor cells in immunodeficient mice. (b) Xenografting of CAR T cells for *in vivo* testing in immunodeficient mice. (c) Diagram of flow cytometry, a common procedure to count the number of cells expressing different cell surface proteins [6].

Additional tests used to characterize how well the cytokine circuit functions *in vivo* including a cell killing assay, which measures the release of fluorescently tagged apoptotic molecules such as caspase and IP3 in cells [6], shown in Figure 5, measurements of survival rates in mice, tumor volume, and tumor burden, quantified by imaging of photons emitted tumors tagged with fluorescent luciferase protein [8]. To test how well the switch prevents cytokine release syndrome, Raji line human lymphoma cells will be injected intraperitoneally into a SCID-beige mouse model devoid of NK cells to create a CRS model for testing [6], as shown in Figure 6. CRS can be induced by grafting a tumor in a mouse and then injecting large amounts of CAR T cells and GSV in the area [6]. In this part of the experiment,

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IL-2 and other serum cytokine levels, as well as overall mice survival rates, will be used to measure how effective the ON switch is at dealing with CRS [6].

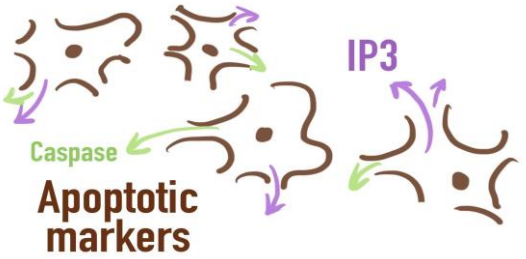

Figure 5. Cell-killing assay that detected the release of apoptotic messengers from dying tumor cells with fluorescent reagents [6].

Figure 6. Intraperitoneal injection of Raji line lymphoma cells, CAR T cells with the drug-gated synNotch circuit, and administering GSV to immunodeficient mice in order to model CRS. Once CRS symptoms appear the ON switch in the synNotch circuit will be tested to see if it can halt secretion of IL-2 and relieve CRS symptoms in the absence of GSV [6].

3. Discussion

3.1. Experimental Rationale

Our experiment applies drug-induced tunable circuits to regulate armored CAR T cells that use cytokine signaling to overcome the immunosuppressive TME. This study aims to mitigate CRS and other adverse side effects of immune system modulation. By designing and implementing a CAR-independent synNotch cytokine circuit, we aim to directly reduce the effects of CRS and enhance control over T cell activation without altering the CAR.

3.2. Benefits

This method is superior to several alternative approaches because CAR T cell activity is readily inducible and extrinsically controlled. IL-2 also has immunosuppressive effects on Treg cells, and controlling the level of IL-2 expression is vital in reducing this effect [8]. Compared to natural receptor signaling systems, orthogonal synthetic systems minimize crosstalk (for example, with the JAK-STAT signaling pathway involved in cytokine signaling) and interference with other pathways by avoiding the use of natural receptors or signaling molecules [4]. Designing a drug-gated receptor is relatively simple, and the antiviral drugs that induce it have known safety profiles, pharmacokinetics, and are FDA-approved, simplifying clinical application [6].

3.3. Alternative Approaches

Some alternative approaches to treat solid tumors include the application of focused ultrasound (FUS) on cytokines, the usage of synNotch, and combinatorial methods. Short pulses of FUS stimulation could target cytokines at a desired location and time, acting as a gene switch on the proliferation of cytokine

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secretion in targeting tumor cells [7]. However, this method is more impractical and less versatile than drug-induced circuits, as it is only controlled as an ON switch and cannot be turned off. The synNotch receptor by itself, combined with an artificial cytokine signaling pathway, significantly reduces off-target toxicity and amplifies cytokine secretion [8]. However, it is self-regulating and cannot be controlled directly. Unmodified synNotch receptors also exhibit ligand-independent activation and benefit from an additional control layer to avoid potential cases of off-target toxicity [10]. One alternative cytokine circuit we considered is a FUS-controlled gene circuit with the IL-2 transgene. Under FUS stimulation, CAR T cells could be activated in a localized time-dependent manner, providing a switch of IL-2 proliferation without needing ligand binding. However, as mentioned above, the limited flexibility of the FUS gene switch makes it a less suitable system.

4. Conclusion

We hope this study will improve synNotch cytokine circuits by adding an ON switch as an additional control layer. An essential limitation of this experiment is the difficulty in creating an OFF synNotch receptor, as the structure of the Notch protein does not lend itself to a split polypeptide configuration used in previous studies to construct drug-gated OFF switches [4,6,8]. However, there are other types of synthetic receptors, proteases, or other proteins that can be regulated by clinically available drugs that have not been explored and could be used as an OFF switch in the future. As part of the next generation of emerging CAR T cell therapies, drug-inducible circuits will make CAR T cells safer, more effective, and more specific in treating cancer patients, but they require more research in order to fully explore its many potential use cases.

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David Drew, Xinyan Li, Michael Su, and Qingmei Wang contributed equally to this work and should be considered co-first authors.

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