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TITLE: ENGINEERING NEXT GENERATION CAR T CELLS TO TREAT PEDIATRIC AML: ENHANCING SAFETY THROUGH DYNAMIC CONTROL AND SPECIFICITY.

PRINCIPAL INVESTIGATOR: Wendell Lim

CONTRACTING ORGANIZATION: The Regents of the University of California, San Francisco

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WENDELL A. LIM, PHD

E-Mail: wendell.lim@ucsf.edu

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

REGENTS OF THE UNIVERSITY OF
CALIFORNIA, SAN FRANCISCO
CONTROLLER'S OFFICE
1855 FOLSOM ST STE 420
SAN FRANCISCO CA 94103-4241

8. PERFORMING ORGANIZATION REPORT NUMBER

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Acute myeloid leukemia (AML) is curable in less than 30% of patients. Immunotherapeutic approaches have changed the landscape of treatments for patients with B-lineage malignancies but have not yet been effective in myeloid malignancies due to concerns over on-target/off-tumor effects on healthy myeloid cells. No AML-specific cell surface antigens have been identified and using conventional chimeric antigen receptor (CAR) T cell therapy to target myeloid antigens would likelylead to life-threatening myelosuppression. In the Lim lab, we have developed two novel receptors that we believe have the potential to overcome the obstacles of on-target/off-tumor toxicity. Our goal is to improve the treatment of AML by developing next generation immunotherapy with enhanced AML specificity and decreased toxicity. This is a collaborative venture between a leader in T cell therapy engineering (Lim) and a leader in childhood leukemia (Loh). We have made significant progress towards accomplishing the goals of this project. We have taken two approaches using recently developed, novel receptors to generate a CAR T cell with titratable cytotoxic activity and a dual receptor, AND-gate CAR T cell. We have developed synthetic cellular circuitry to 1) titrate cytotoxic activity of a CAR T cell and 2) to target abnormal combinations of cell surface antigens that are specific to leukemic cells and will spare toxicity to healthy myeloid cells. Over the last year we have focused on studying the dual receptor. Preliminary results show that this receptor is able to achieve titratable and reversible control of CAR T cell activity. In vitro this circuit achieves tumor clearance similar to the constitutive CAR expression and shows less T cell differentiation as well as less exhaustion. These characteristics have been positively correlated with better in vivo performance in mice and humans.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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TABLE OF CONTENTS

1. INTRODUCTION:

Pediatric myeloid hematopoietic malignancies represent a spectrum of diseases that are particularly challenging to treat. The majority of pediatric myeloid leukemias are acute myeloid leukemia (AML). Although progress has been made in the treatment of pediatric AML, the 5-year survival rate is still approximately 50%.[2] There is an urgent need to develop more effective and less toxic treatments for this deadly disease. Chimeric Antigen Receptor (CAR) T cell therapies have emerged as a powerful class of anti-cancer therapeutics, particularly for B-cell acute lymphoblastic leukemia (B-ALL), where unprecedented rates of remission have been achieved in the multiply relapsed and refractory population. CARs are a synthetically engineered receptor that are expressed in an autologous T cell and allow for re-direction of cytotoxic T cell activity towards an antigen of choice. presents a particularly attractive candidate for T cell therapy for a number of reasons: extensive knowledge of AML cell surface expression exists, relative ease of sampling tumor from peripheral blood draws or bone marrow aspirates, and a natural preference for T cells to home to hematologic organs such as the blood, bone marrow and lymph nodes. However, there are no leukemia-specific surface antigens in AML. Although these CAR T cells are capable of effectively eradicating AML in vitro, they would likely lead to profound and potentially fatal myelosuppression via on-target/off-tumor myeloid progenitor cell depletion.

2. KEYWORDS:

Acute myeloid leukemia, immunotherapy, pediatric oncology, chimeric antigen receptor T cells, synthetic notch receptor, hematologic malignancies, immune-oncology, synthetic biology

3. ACCOMPLISHMENTS:

What were the major goals of the project?

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Specific Aim 1(specified in proposal)	Proposed Timeline	Percent Completed
Major Task 1: Engineer anti-AML CAR T cells with a controllable ON/OFF-switch	Months	50%
Subtask 1: ON/OFF CAR scFv design and cloning	1-2	50%
Subtask 2: Confirm CAR T cell recognition and cytotoxic activity in vitro in AML cell lines and patient samples	3-6	100%
Subtask 3: Assay toxicity in vitro in human cord blood and bone marrow cells	6-9	50%

Subtask 4: In vivo assay to assess AML cell clearance and healthy myeloid toxicity	9-18	10%
Major Task 2: Generate dual-receptor, AND-gate T cells with enhanced myeloid leukemic specificity		40%
Subtask 1: design and clone AND-gate T cell circuitry with CD33 synNotch and aberrant antigen B (CD123, CD15, CD64, CD19) CAR	1-2	60%
Subtask 2: design and clone AND-gate T cell circuitry with CD33 or CD123 synNotch and CD33 or CD123 CAR to target antigens that are over-expressing AML cells	3-4	40%
Subtask 3: design and clone AND-gate T cell circuitry with CD33 synNotch and stress antigen B (CD47 or NKG2D ligand) CAR	5-6	30%
Specific Aim 2	**Proposed Timeline**	**Percent Completed**
Major Task 3: test dual-receptor, AND-gate T cells for AML specificity and cytotoxicity as well as healthy myeloid toxicity		25%
Subtask 1: Confirm dual-receptor, AND-gate T cell recognition and cytotoxic activity in vitro in AML cell lines and patient samples	6-12	25%
Subtask 2: Assay toxicity in vitro in human cord blood and bone marrow cells	12-15	0%
Subtask 4: In vivo assay to assess AML cell clearance	15-24	0%

What was accomplished under these goals?

Major Conclusions:

- Major Task 1: We have generated a novel ON/OFF switch which is controlled by a clinically safe small molecule, Nirogacestat. Here we use a synNotch receptor to induce expression of a CAR. Nirogacestat is a gamma-secretase inhibitor, that blocks cleavage and output of the synNotch receptor. Preliminary results show that this can be used to achieve titratable and reversible control of CAR T cell activity. In vitro this circuit achieves tumor clearance similar to the constitutive CAR expression and shows less T cell differentiation as well as less exhaustion. These characteristics have been positively correlated with better in vivo performance in mice and humans.
- Major Task 2: No major change since last year.
- Major Task 3: No major change since last year

Major activities, specific objectives, significant results or key outcomes:

On Specific Aim 1, we have are working rapidly towards completion of the ON/OFF switch: In addition to the proposed split CAR proposed, we have also developed an additional, more effective switch with great promise for the treatment of AML. This switch is controlled by Nirogacestat, a small molecule that was fast tracked for FDA approval in 2018. Since last year we have received IACUC protocol approval to proceed with planned in vivo experiments using Nirogacestat.

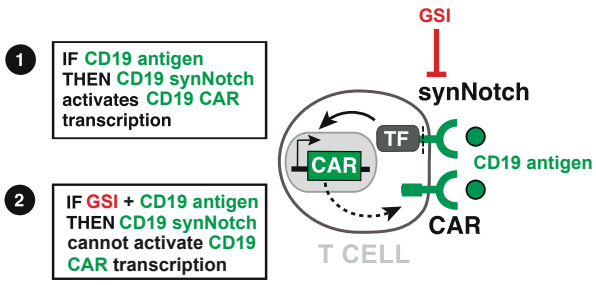

Figure 1: Titratable approach for cytotoxic therapy administration.

Our approach uses an autoregulatory synthetic circuit that is capable of dynamically controlling chimeric antigen receptor (CAR) cytotoxic T cell activity. This T cell circuit is dependent on using two receptors to recognize the target disease antigen – first a synthetic notch (synNotch) receptor recognizes the B-cell specific antigen, CD19, on the surface of B-cell leukemia and lymphoma cells, and in response, induces the gene expression of a CAR that also recognizes the target antigen, CD19 (Figure 1.1). This circuit is capable of autoregulation – the cytotoxic activity enabled by the CAR is only induced when the synNotch receptor first detects cells with CD19. Conversely, once malignant cells expressing CD19 cells are killed, CAR expression and cytotoxic activity will autonomously decrease in response to decreasing antigen burden. This two-receptor circuit has two main advantages over the conventional, constitutively expressed CAR T cell: 1) in the absence of antigen, CAR is not expressed, minimizing tonic signaling during CAR T cell manufacturing and expansion (tonic signaling is known to lead to T cell exhaustion), and 2) in the presence of antigen, CAR expression is dependent on antigen detection by synNotch and therefore CAR expression is dynamic, proportional to antigen load, and expressed only at the level required to clear antigen, features that are likely to lead to reduced T cell exhaustion in vivo (and longer overall persistence of T cells) and reduced toxic adverse side effects, such as strong cytokine release syndrome.

In addition, this dual receptor circuit can be controlled by an exogenous drug – the synNotch receptor can be tightly regulated by the class of small molecule inhibitors, gamma secretase inhibitors (GSI) (Figure 1.2). This regulation is titratable and user-controlled, so that the user can fine-tune the amount of cytotoxicity that the CAR T cell imposes. Importantly, the OFF-switch is reversible. Upon removal of the GSI, cytotoxic activity of the T cell resumes. This has the potential to be used to enhance the safety of CAR T cells.

We developed a CAR T cell circuit that allows for exquisite control over cytotoxic activity (Figure 1.1). Specifically, we used a synNotch receptor that is specific for the CD19 antigen. Upon binding of synNotch to the CD19 antigen, proteolytic cleavage by gamma secretase induces release of the intracellular orthogonal transcription factor which drives expression of a CD19 CAR. In the absence of CD19 antigen, proteolytic cleavage does not occur, CD19 CAR is not expressed and there is no cytotoxic T cell activity. In the presence of CD19 antigen, CD19 CAR is expressed and cytotoxic activity is directed towards target cells expressing the CD19 antigen.

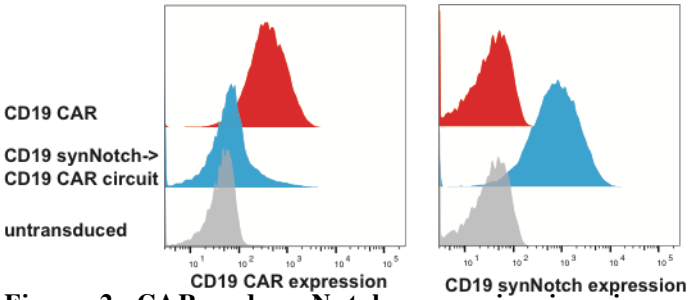

Figure 2: CAR and synNotch expression in primary human CD8 T cells. The CD19 synNotch -> CD19 CAR circuit shows no CAR expression when unstimulated with the CD19 antigen.

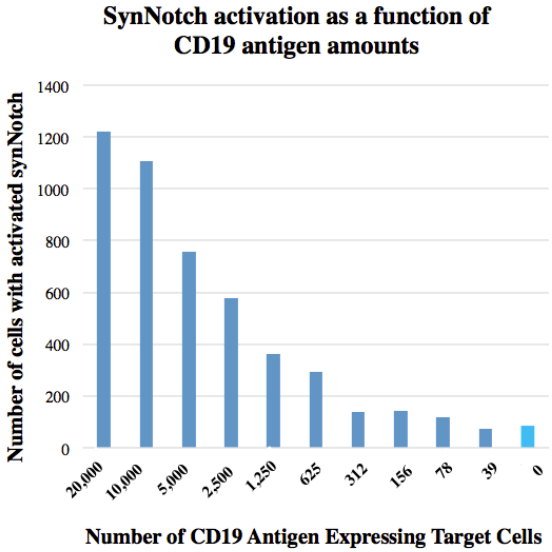

Figure 3: CD19 SynNotch can detect very low cell density and respond in a dose-dependent fashion. The same number of T cells expressing a CD19 synNotch and a BFP reporter for synNotch activity were cocultured with decreasing numbers of CD19 antigen expressing cells. The number of BFP positive cells was used as a readout of synNotch activation.

We first confirmed that the CD19 CAR and CD19 synNotch were expressed in primary human CD8 T cells using flow cytometry analysis. Notably, in CAR T cells expressing the autoregulatory circuit, no CD19 CAR expression was observed in the absence of CD19 antigen (Figure 2). Minimal residual disease, or very low level disease (between 0.01%-5%) disease burden of nucleated bone marrow cells is the number one reason for relapse and is associated with nearly 100% rate of relapse. In order to determine if SynNotch can detect very low cell density disease, we exposed CD19 synNotch reporter T cells to decreasing amounts of CD19 positive target cells. In response to decreasing antigen burden, CAR expression correspondingly decreased, suggesting autoregulatory capability (Figure 3). Further, CD19 synNotch was able to detect very low numbers of CD19 expressing cells.

Constitutive or chronic cell signaling due to the CAR expression without exposure to the cognate antigen, known as CAR tonic signaling, has been shown to have deleterious impact on CAR T-cell effector function and survival [17,18]. We therefore assessed CAR tonic signaling by measuring the level of CD69, a marker of T cell activation. We observed low levels of CD69 expression in the CD19 CAR T cells in the absence of CD19 antigen suggesting tonic signaling (Figure 4). Remarkably, the autoregulatory CAR T cell exhibited no tonic signaling. This is consistent with

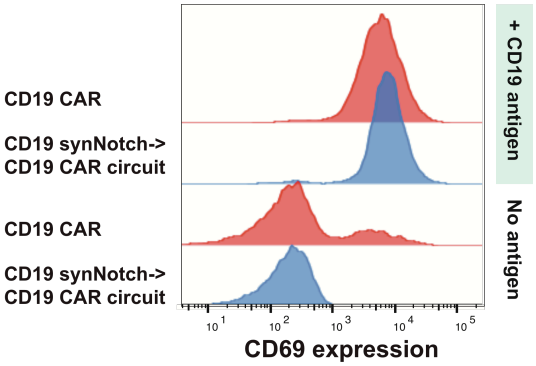

Figure 4: CD19 synNotch -> CD19 CAR circuit shows full activation potential with no tonic signaling. Primary human CD8 T cells were stained for CD69 , a marker for activation with or without CD19 antigen stimulation.

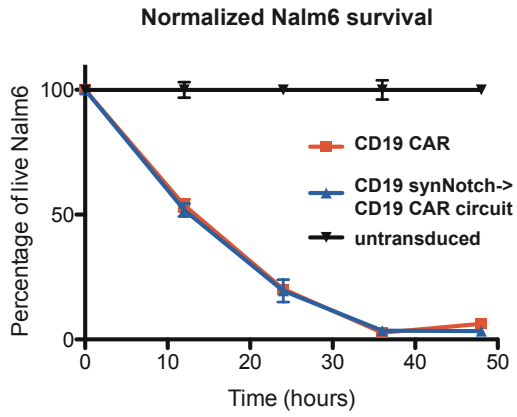

Figure 5: CD19 synNotch -> CD19 CAR circuit shows similar cytotoxicity as constitutive CD19 CAR. Primary human CD8 T cells were cocultured with Nalm6 (CD19 positive). Nalm6 survival was normalized to untransduced T cells coculture for each time point.

prior data demonstrating no expression CAR expression. Upon exposure to the CD19 antigen, both the CD19 CAR and CD19 synNotch-> CD19 CAR circuit showed similar activation of the T cell response. Thus, the autoregulatory CAR T cell and the constitutive CD19 CAR T cell exhibit comparable levels of activation when exposed to CD19 antigen, however, the autoregulatory CAR T cell exhibits significantly less tonic signaling in the absence of antigen. To further characterize the functionality of the autoregulatory CAR T cell, we co-cultured the engineered T cells with Nalm6 cells (a patient derived B-ALL cell that expresses endogenous CD19) to quantify T cell cytotoxicity. The autoregulatory CAR T cell and the constitutively active CAR T cell both exhibited potent and specific cancer cell line killing over 48 hours (Figure 5).

We conclude that autoregulatory CAR T cell have potent, specific killing capacity but minimal tonic signaling in the absence of antigen.

Tonic signaling has been shown to increase exhaustion and induce effector T-cell differentiation resulting in poor *in vivo* performance. Central memory T cells and stem cell-like memory T cells are known to promote sustained proliferation and persistence after T-cell therapy. Additionally, lack of CAR-T cell *in vivo* persistence was due to a lower percentage of naïve T cells vs. effector memory T cells prior to *in vivo* delivery as *ex vivo* cultivation and expansion of the T cell skews the proportions of these subpopulations.

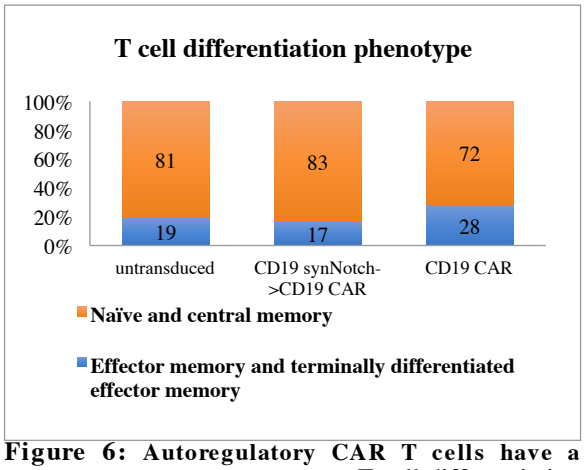

Figure 6: Autoregulatory CAR T cells have a less differentiated phenotype. T cell differentiation phenotype was assessed using CCR7 and CD45RA cell surface markers.

To evaluate the extent of differentiation in our engineered T cells, we used CCR7 and CD45RA cell surface markers to distinguish between the different states of differentiation: naïve ($CD45RA^{+}CCR7^{+}$), central memory ($CD45RA^{-}CCR7^{+}$), effector memory ($CD45RA^{-}CCR7^{-}$), and terminally differentiated effector memory ($CD45RA^{+}CCR7^{-}$). Strikingly, autoregulatory CAR T cells showed less differentiation and a more naïve/central memory phenotype compared to the constitutive CD19 CAR (Figure 6).

Importantly, this suggests that autoregulatory CAR T cells may exhibit decreased exhaustion, enhanced durability and ultimately, improved efficacy.

No major change since last year has been done for Major tasks 2 and 3

Stated Goals Not Met

We have not been able to test Nirogacestat in vivo as the price has been prohibitive so far using our regular drug suppliers ($1,000/mouse/experiment). We are in contact with Spring Works therapeutics, the company synthetizing FDA approved Nirogacestat to obtain free or highly discounted product. They are very interested in the project.

What opportunities for training and professional development has the project provided?

Multiple training opportunities are provided to those students and postdocs involved in this project. In addition to close one-on-one mentoring, the trainees participate and present in several regular meetings – the Lim Lab group meeting, the UCSF Systems and Synthetic Biology Center monthly meeting, and the UCSF Cell Therapy club (once a month). This gives them a good exposure in quantitative biology, cell engineering, and translational medicine. The Center for Systems and Synthetic Biology (of which I am Director) also offers training courses for professional development, in terms of helping trainees with grant writing, preparing for lab management, etc. We also support workshops for increasing diversity among trainees. There are also ample opportunities for trainees to mentor high school, undergraduate or rotation students. We are developing IDPs for all of our trainees in which we will regularly review goals for the year, as well as plan for their evolving long- term career objectives.

How were the results disseminated to communities of interest?

Disseminating the results of our research to the community is integral to keep the field moving forward. Our efforts include:

Education. Dr. Lim has developed programs on cell engineering and therapy with local K-12 schools. The iGEM summer research program is a long-term partnership with Biotechnology classes in the San Francisco Unified School District, which has been featured on TV, the SF Chronicle and the NYTimes. Top students come to the lab and work on cell engineering projects that they help to develop. We have also developed demonstrations on cell engineering for middle school biology classes and high school science teachers.

Exhibits/Demos. We have presented demonstrations on cell engineering and therapy at the Exploratorium of San Francisco.

Fundraising. We are central participants in the UCSF Capital Campaign, one of the largest fundraising efforts ever set by a U.S. university ($5 billion). The Lim Lab has led top donors through an exercise engineering “self-driving” cells from a person’s own immune system to kill cancer.

What do you plan to do during the next reporting period to accomplish the goals?

- Initiate in vivo studies for AND-gate and ON/OFF switch circuits
- Test and optimize AND-gate circuits to enhance dual antigen targeting specificity
- Test ON/OFF CAR T cell on patient derived AML cell lines in vitro and in vivo
- Continue optimization of split CAR ON/OFF switch using FDA approved small molecules

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The Lim lab has developed several innovative strategies to tackle this obstacle, including designing an ON/OFF switch CAR and a dual-receptor, AND-gate CAR T cell that enhances on-target specificity. We have made significant progress towards implementing these techniques to tackle AML using cellular therapies. The completion of this project could result in new "living drugs" that will harness never before used technologies to advance the field of immunotherapy for AML.

What was the impact on other disciplines?

This invention improves the safety of engineered cellular therapies. There are already several engineered cellular therapies that have been approved by the FDA (axicabtagene ciloleucel, tisagenlecleucel) and a staggering number in clinical trials. This invention could be used to enhance the safety and efficacy of all existing and future cellular therapies by providing a safe and effective means to control the cytotoxic activity of cellular therapies.

What was the impact on technology transfer?

This invention improves the safety of engineered cellular therapies. There are already several engineered cellular therapies that have been approved by the FDA (axicabtagene ciloleucel, tisagenlecleucel) and a staggering number in clinical trials. This invention could be used to enhance the safety and efficacy of all existing and future cellular therapies by providing a safe and effective means to control the cytotoxic activity of cellular therapies.

Cell Design Labs was founded based on the synNotch receptor and split CAR ON-switch technology. Cell Design Labs was acquired by Kite Pharma who was acquired by Gilead Sciences, Inc. The technology has also been licensed out to other inventors by Gilead Sciences, Inc.

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Nothing to Report

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

Nothing to Report

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Wendell Lim*
Project Role:	*PD/PI*
Researcher Identifier (e.g. ORCID ID):	*0000-0003-4052-8056*
Nearest person month worked:	*0.4*
Contribution to Project:	*Dr. Lim is responsible for overseeing all aspects of the project.*
Funding Support:	*Howard Hughes Medical Institute*
Name:	*Mignon Loh*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0003-4099-4700*
Nearest person month worked:	*0.3*
Contribution to Project:	*Dr. Loh is responsible for co-overseeing all aspects of this project, particularly those aspects associated with AML cell lines and AML xenograft models.*
Funding Support:	*Leukemia and Lymphoma Society, Developmental and Hyperactive Ras Tumor SPORE, Cookies of Kids' Cancer, Dana-Farber Cancer Institute, Incyte Corporation, NIH/NCI, Alex's Lemonade Stand Foundation, NIH/NIGMS, St. Bladrick's Consortium*
Name:	*Ashley Koegel*
Project Role:	*Clinical Fellow*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-6722-9852*
Nearest person month worked:	*6*
Contribution to Project:	*Dr. Koegel has performed work in the area of synthetic and molecular biology as well as testing of primary human T cell circuits against patient derived AML cell lines.*
Funding Support:	*California Institute of Regenerative Medicine, Alpha Stem Cell Clinic Fellowship*
Name:	*Michael Broeker*
Project Role:	*Staff research associate III*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*9*
Contribution to Project:	*Assistant Specialist support in cell culture and cell analysis.*
Funding Support:	*Defence Advanced Research Projects Agency; Cell Design Institute*

Name:	*Milos Simic*
Project Role:	*Postdoctoral fellow*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*6*
Contribution to Project:	*Dr. Simic has performer work in the area of synthetic and molecular biology as well as testing of primary human T cell circuits*
Funding	*N/A*
Name:	*Jason Duecker*
Project Role:	*Junior specialist*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*6*
Contribution to Project:	*Mr. Duecker has helped characterize the human T cell circuits*
Funding	*N/A*
Name:	*Margo Wohlfeil*
Project Role:	*Staff research associate*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*6*
Contribution to Project:	*Ms. Wohlfiel replaced Ella Melnick when Ms. Melnick began graduate school at the University of California, Berkeley. She has banking myeloid tumor samples in the tissue bank and performing drug screens.*
Funding	*N/A*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: