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TITLE: Precision Combinatorial Immunotherapeutic Targeting of Cytokine Receptor Kinase Signaling in CRLF2-Rearranged ALL

PRINCIPAL INVESTIGATOR: Sarah Tasian

CONTRACTING ORGANIZATION: **Children's Hospital of Philadelphia, PA**

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6. AUTHOR(S)

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E-Mail:

5d. PROJECT NUMBER

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5f. WORK UNIT NUMBER

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

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PHILADELPHIA PA 19104-4319

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14. ABSTRACT

Ph-like ALL is a high-risk subset of B-ALL defined by an activated kinase gene expression profile similar to that of BCR-ABL1-rearranged (Ph+) ALL and driven by a diverse range of genetic alterations that activate cytokine receptor signaling pathways. Children, AYAs, and older adults with Ph-like ALL have >60% relapse risk and experience significant leukemia-associated mortality. Approximately 50% of Ph-like ALL cases harbor rearrangements in CRLF2 (CRLF2-R) and frequent concomitant JAK2 point mutations. In addition to patients with Ph-like ALL, CRLF2 rearrangements (usually P2RY8-CRLF2 fusions) with JAK2 point mutations occur in approximately 60% of children and AYAs with trisomy 21/Down Syndrome-associated ALL (DS-ALL) and also induce hyperactive JAK/STAT signaling. Children with DS-ALL have substantial toxicity with chemotherapy and inferior clinical outcomes. CD19CART immunotherapy has proven highly successful at inducing remissions in 80-90% of patients with relapsed/refractory ALL. However, emerging data indicates that up to 50% of children and AYAs will relapse, most within a year. As an alternative strategy, the Fry laboratory developed CAR constructs targeting the TSLPR (encoded by CRLF2) and demonstrated potent in vivo activity of T cells transduced with anti-TSLPR CAR constructs (TSLPRCART) in CRLF2-R Ph-like ALL PDX models generated by the Tasian laboratory.

15. SUBJECT TERMS
None listed.

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1. INTRODUCTION:

Ph-like ALL is a high-risk subset of B-ALL defined by an activated kinase gene expression profile similar to that of *BCR-ABL1*-rearranged (Ph+) ALL and driven by a diverse range of genetic alterations that activate cytokine receptor signaling pathways. Children, AYAs, and older adults with Ph-like ALL have >60% relapse risk and experience significant leukemia-associated mortality. Approximately 50% of Ph-like ALL cases harbor rearrangements in *CRLF2* (*CRLF2*-R) and frequent concomitant *JAK2* point mutations. In addition to patients with Ph-like ALL, *CRLF2* rearrangements (usually *P2RY8-CRLF2* fusions) with *JAK2* point mutations occur in approximately 60% of children and AYAs with trisomy 21/Down Syndrome-associated ALL (DS-ALL) and also induce hyperactive JAK/STAT signaling. Children with DS-ALL have substantial toxicity with chemotherapy and inferior clinical outcomes. CD19CART immunotherapy has proven highly successful at inducing remissions in 80-90% of patients with relapsed/refractory ALL. However, emerging data indicates that up to 50% of children and AYAs will relapse, most within a year. As an alternative strategy, the Fry laboratory developed CAR constructs targeting the TSLPR (encoded by *CRLF2*) and demonstrated potent *in vivo* activity of T cells transduced with anti-TSLPR CAR constructs (TSLPRCART) in *CRLF2*-R Ph-like ALL PDX models generated by the Tasian laboratory. Based on our promising preclinical data, a phase 1 clinical trial of TSLPRCART for children and AYAs with relapsed CRLF2/TSLPR-overexpressing ALL will soon open at the NIH. TKIs and CART immunotherapies have the potential to act synergistically in acute leukemias via co-targeting of oncogenic pathways using two distinct approaches: one (CART) targeting a cell surface cytokine receptor protein and the other (TKI) targeting critical receptor-mediated and intracellular kinase signaling pathways. Furthermore, combining multi-targeted CAR T cells with TKIs is strategically analogous to the paradigm of non-cross-resistant cytotoxic chemotherapy regimens that is required to achieve cure in children with ALL. This application is directly relevant to FY18 PRCRP Topic Areas of (1) Blood Cancer, (2) Immunotherapy, and (3) Cancer in Children, Adolescents, and Young Adults. *The primary hypothesis of this proposal is that durable remissions in patients with CRLF2-R Ph-like ALL or DS-ALL can be achieved using rationally combined immune and molecular kinase therapies that target critical and necessary signaling pathways in malignant cells.* The project is divided into the following Aims:

Aim 1. Develop combinatorial CAR constructs targeting TSLPR plus CD19 and/or CD22 and test the anti-leukemia efficacy of multi-targeted CARTs against *CRLF2*-R ALL in children, adults, and DS patients.
Aim 2. Determine the preclinical efficacy of multi-specific CARTs and kinase inhibitors against *CRLF2*-R ALL.
Aim 3. To delineate the impact of DS-associated immunodeficiency and aging on the potency of CART generated from of patients with DS-ALL and to determine functionality of autologous T cell transduction for clinical immunotherapy with CARTs.

Study Design: These complementary and imminently clinically-translatable studies will test and validate the potential treatment efficacy of multi-targeted CART immunotherapy and/or kinase inhibition in multiple subtypes of *CRLF2*-R ALL characterized by TSLPR overexpression and hyperactivation of cytokine signaling, as well as increase our understanding of T cell functionality and therapeutic potential in patients with trisomy 21. In this work, we will develop synergistic, non-

overlapping treatment strategies to improve leukemia remission durability and to mitigate development of immunotherapeutic resistance in three very high-risk subtypes of B-ALL.

2. KEYWORDS:

Acute lymphoblastic leukemia, immunotherapy, kinase, cytokine receptor, thymic stromal lymphopoietin, chimeric antigen receptor, t cell

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Develop combinatorial CAR constructs targeting TSLPR plus CD19 and/or CD22 and test the antileukemia efficacy of multi-targeted CARTs against CRLF2-R ALL.

Major task 1: design of bispecific TSLPR x 19/22 CAR constructs & T cell manufacturing

Major task 2: new ALL PDX model creation

- expand previously established childhood/adolescent *CRLF2*-R Ph-like ALL for subsequent experimental therapeutic studies
- establish 2-4 primary xenograft models of adult *CRLF2*-R Ph-like ALL and expand in secondary recipients for subsequent experimental therapeutic studies
- establish 2-4 primary xenograft models of DS-ALL and expand in secondary recipients for subsequent experimental studies

Major task 3: *in vitro* testing of TSLPRCARTs with *CRLF2*-R ALL cell lines MUTZ5 and MHH-CALL-4

- cytokine production assays (IL-2, IFN-gamma)
- cytotoxicity assays (Cell Titer Glo) with ALL cell lines + CAR T cells +/- TKI

Major task 4: in vivo testing of TSLPRCARTs

- preclinical testing of TSLPRCART and ruxolitinib monotherapies in bioluminescent CRLF2-R cell line MUTZ5 xenograft models
- preclinical testing of TSLPRCART + ruxolitinib in bioluminescent *CRLF2*-R cell line MUTZ5 xenograft models
- preclinical testing of TSLPRCARTxCD19 in bioluminescent *CRLF2*-R cell line MUTZ5 xenograft models
- preclinical testing of TSLPRCARTxCD22 in bioluminescent *CRLF2*-R cell line MUTZ5 xenograft models

Major task 5: TSLPR surface density quantification of phase 1 clinical trial ALL patient samples
• NIH TSLPRCART trial to open in Q2 2021; estimated patient accrual over 2-3 years
• Flow cytometric immunophenotyping of bone marrow TSLPR surface antigen density at study entry (and relapse if applicable)

Milestones achieved: identification of optimal combinatorial TSLPRCART(s) in vivo in CRLF2-R ALLs, final data analysis

Specific Aim 2: Determine the preclinical efficacy of multi-specific CARTs and JAKi against *CRLF2*-R ALL

Major task 1: *in vivo* testing of TSLPRCARTs/TKIs peds ALL PDXs
• preclinical testing and efficacy comparison of TSLPRCART, CD19CART, CD22CART, TSLPRxCD19CART, TSLPRxCD22CART in 2-4 childhood *CRLF2*-R ALL PDX models (see tables below for cohort and total mouse numbers)
• preclinical testing and efficacy comparison of TSLPRCART + ruxolitinib in 2-4 childhood CRLF2-R ALL PDX models

Major task 2: in vivo testing of TSLPRCARTs/TKIs adult ALL PDXs
• preclinical testing and efficacy comparison of TSLPRCART, CD19CART, CD22CART, TSLPRxCD19CART, TSLPRxCD22CART in 2-4 adult CRLF2-R ALL PDX models
• preclinical testing and efficacy comparison of TSLPRCART + ruxolitinib in 2-4 adult ALL CRLF2-R ALL PDX models

Major task 3: in vivo testing of TSLPRCARTs/TKIs DS-ALL PDXs
• preclinical testing and efficacy comparison of TSLPRCART, CD19CART, CD22CART, TSLPRxCD19CART, TSLPRxCD22CART in in 2-4 CRLF2-R DS-ALL PDX models
• preclinical testing and efficacy comparison of TSLPRCART + ruxolitinib in 2-4 DS-ALL CRLF2-R ALL PDX models

Major task 4: ALL TKI effects in vitro and in vivo
• Normal T cell donor flow cytometric immunophenotyping (pediatric Ph-like, adult Ph-like, children with trisomy 21/DS)
• T cell and ruxolitinib coincubation studies: T cell subset flow immunophenotyping), cell death (Annexin-V/PI flow cytometry, cell counting), cell proliferation (CSFE assays)
• TSLPRCART and ruxolitinib coincubation studies: cytokine quantification, effects on proliferation

Milestone(s) Achieved: selection of optimal TSLPRCART/TKI therapy in vivo in each CRLF2+ ALL subtype, final data analysis

Specific Aim 3: To delineate the impact of immunodeficiency associated with Down Syndrome and aging upon T transduction efficiency and functionality of CARTs.

Major task 1: B-ALL adult patient T cell functionality for CAR engineering

• Identification of patient samples at Penn (n=20-30)
• T cell flow immunophenotyping, analysis of senescence biomarkers, pre/post-treatment Treg suppression
• RNAseq to measure TCR repertoire diversity before and after T cell expansion and CAR construct transduction

Major task 2: DS-ALL T cell functionality for CAR engineering
• Identification of patient samples at CHOP and Colorado (n=6-12)
• T cell flow immunophenotyping, analysis of senescence biomarkers, pre/post-treatment Treg suppression
• RNAseq to measure TCR repertoire diversity before and after T cell expansion and CAR construct transduction

Milestone(s) Achieved: elucidation of T cell biologic features that contribute to CAR T cell success and failure, final data analysis.

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Specific Aim 1 Progress to date:
* *Note bene*: Please note that our research laboratories were closed from March to July 2020 due to institutional research restrictions during the COVID-19 pandemic.

1. Task 1: TSLPR x CD19 construct engineering done with in vitro and in vivo experiments in cell line xenograft models now ongoing.
2. Task 2: Childhood and adult ALL PDX models expansion complete. Seven new DS-ALL PDX models established in primary mice and have now completed secondary expansion.
3. Task 3: MUTZ5 in vitro cytotoxicity and cytokine production pilot studies completed.
4. Task 4: TSLPRCART and ruxolitinib in vivo testing in MUTZ5 xenograft models for dose and timing optimization. Two additional PDX model studies now completed. Some correlative biology assessments in process/results pending.
5. Task 5: Phase 1 TSLPR CAR T cell clinical trial is not yet open due to administrative delays at the NIH. Anticipated protocol submission to IRB in Q1 2021.

Specific Aim 2 Progress to date:
* *Note bene*: Please note that our research laboratories were closed from March to July 2020 due to institutional research restrictions during the COVID-19 pandemic.

1. Task 1: PDX model studies ongoing with TSLPR CAR T cells and ruxolitinib. Bispecific TSLPRxCD19 and TSLPRxCD22 CAR constructs made. Expertimental

troubleshooting ongoing in antigen-combination Nalm-6 xenograft models for in vivo testing of bispecific CAR T cells.
2. Task 2: All adult PDX models established, no in vivo testing yet
3. Task 3: All DS-ALL PDX models now established. In vivo testing of ruxolitinib now ongoing.
4. Task 4: Normal T cell studies with ruxolitinib completed; in process of obtaining normal T cells from DS patients at Colorado (unable to access easily at CHOP after much investigation).

Specific Aim 3 Progress to date:
* *Note bene*: Please note that our research laboratories were closed from March to July 2020 due to institutional research restrictions during the COVID-19 pandemic.

1. Task 1: Identified normal T cell donor sources at CHCO and CHOP; no experimental studies done yet. New single-cell/RNAseq studies pending in sorted CAR T cells from above xenograft mice studies to assess T cell expression changes in vivo over time.
2. Task 2: Identifying new collaborator at CHCO for normal DS T cell specimens. Access to DS samples at CHOP has been challenging.

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

Three post-doctoral fellows and one junior faculty member are actively leading hands-on work for this award in our respective laboratories at Children's Hospital Colorado and Children's Hospital of Philadelphia. Two young research technicians (who will soon attend medical school or graduate school) are also assisting with experiments in our labs. Trainees are encouraged to present their work formally at lab meetings and scientific symposia, as well as to submit abstracts to and attend national hematology meetings. Trainees meet regularly one-on-one with their mentors Drs Fry and Tasian and regularly attend scientific seminars/workshops at their institutions to increase their knowledge and continue their career development.

How were the results disseminated to communities of interest?

Research-in-progress and results have been shared locally with other scientific researchers at our institutions. Scientific abstracts of some of this work were submitted to the Biennial Childhood Leukemia & Lymphoma Symposium/annual iBFM 2020 meeting (postponed to 2021 due to the viral pandemic) and to the AACR 2020 annual meeting (virtual meeting poster presentation).

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

We will continue to make experimental progress to achieve the aims of the award according to the proposal timeline. We will continue to submit abstracts of our work and will plan research manuscript writing of initial results in the next year of this award.

4. **IMPACT:**

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

We have made important discoveries about the impact of the JAK inhibitor ruxolitinib upon normal T cell and TSLPR CAR T cell function *in vitro* and *in vivo* in our leukemia studies. These preclinical results will definitively impact our design of and planning for clinical trials testing new precision medicine treatments for patients with *CRLF2*-rearranged ALL. In addition to our established Ph-like ALL models, we have also successfully developed several important new mouse models of pediatric Down Syndrome-associated ALL that provide a critical resource for this project and could also be shared with the greater scientific community in the future.

What was the impact on other disciplines?

Results from our work have broad implications for studies of other cellular immunotherapies and tyrosine kinase inhibitors in human cancer models and clinical trial development.

What was the impact on technology transfer?

Nothing to report in this reporting period. There is potential for future engagement of biotech company collaboration as we translate results from these preclinical studies to clinical trials.

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

Please note that our research laboratories were closed from March to July 2020 due to institutional research restrictions during the COVID-19 pandemic. Both Fry and Tasian laboratories are now fully operational, albeit at reduced density.

Changes that had a significant impact on expenditures

Due to laboratory shut-downs from March to July 2020 resulting from the COVID-19 pandemic, experimental expenditures were lower than anticipated during the months March-June.

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**

  Journal publications.

Nothing to Report

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

Two abstracts of our work were submitted during the annual reporting period:

1. Bagashev *et al.* Co-Targeting of Thymic Stromal Lymphopoietin Receptor Signaling to Decrease Immunotherapeutic Resistance in *CRLF2*-Rearranged ALL. 12th Biennial Childhood Leukemia and Lymphoma Symposium abstract CLLS20-0053 (May 2020; Valencia, Spain - oral presentation; meeting delayed to March 2021 and will be held virtually due to viral pandemic)

2. Ross *et al.* Multi-Antigen Targeting of CD19, CD22 and TSLPR to Prevent Ph-like ALL Resistance. AACR 2020 annual meeting abstract #3234 (Chicago, Illinois; June 2020 - poster presentation; meeting held virtually due to viral pandemic) https://cancerres.aacrjournals.org/content/80/16_Supplement/3234

Other publications, conference papers and presentations.

Nothing to report.

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

Nothing to report.

- **Technologies or techniques**

  During the course of this project, we have successfully developed several important new patient-derived xenograft (PDX) models of *CRLF2*-overexpressing Ph-like ALL and Down Syndrome-associated ALL. These PDX models are a critical resource for this project for in vivo investigation and could also be shared with the greater scientific community in the future.

- **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: Terry Fry, MD (CHCO)
Project Role: PI
Researcher Identifier (e.g. ORCID ID): 0000-0001-8044-5226
Nearest person month worked: 2.4 Calendar
Contribution to Project: Dr. Fry prepared documentation necessary for initiation of research to be conducted on the proposal and oversees the entire project with Dr. Tasian.

Name: Lillie Leach, BS (CHCO)
Project Role: research technician
Researcher Identifier (e.g. ORCID ID): none
Nearest person month worked: 0.6 Calendar
Contribution to Project: Ms Leach has assisted with TSLPR CAR T cell manufacturing in the Fry laboratory following departure of Dr Ross from CHCO at completion of her post-doctoral fellowship. All animal studies at CHCO commenced after obtainment of ACURO approval.

Name: Sarah Tasian, MD (CHOP)
Project Role: Co-PI
Researcher Identifier (e.g. ORCID ID): 0000-0003-1327-1662
Nearest person month worked: 2.4 Calendar
Contribution to Project: Dr. Tasian prepared documentation necessary for initiation of research to be conducted on the proposal and oversees the entire project with Dr. Fry.

Name: Asen Bagashev, PhD (CHOP)
Project Role: Scientist
Researcher Identifier (e.g. ORCID ID): 0000-0003-9900-8106
Nearest person month worked: 3 Calendar
Contribution to Project: Dr Bagashev is responsible for *in vitro* and *in vivo* studies performed at CHOP in the Tasian laboratory. All animal studies commenced after obtainment of ACURO approval.

Name: Joseph Loftus, BS (CHOP)
Project Role: Research technician
Researcher Identifier (e.g. ORCID ID): none
Nearest person month worked: 3 Calendar
Contribution to Project: Mr Loftus is responsible creation and maintenance of the needed ALL PDX models at CHOP in the Tasian laboratory. All animal studies commenced after obtainment of ACURO approval.

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?

Organization Name: Stand Up to Cancer co-funded by the Emily F Whitehead Foundation *Location of Organization:* New York/California and Pennsylvania
Partner's contribution to the project (identify one or more): We received a two-year Phillip A Sharp Award for Innovation in Collaboration for an extension of these Department of Defense Studies (funded June 2019 to May 2021). This additional funding has primarily supported development and serial transplantation of our Down Syndrome ALL PDX models in costly immunocompromised mice with detailed genetic characterization. There is no budgetary overlap with the Department of Defense award.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Please see detailed reports and allocation of research efforts for Drs Fry and Tasian detailed in our quarterly progress reports and most recent update in October 2020.

QUAD CHARTS:

9. APPENDICES: